NEUROPEPTIDES

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AIMS AND SCOPE

The aim of **NEUROPEPTIDES** will be the rapid publication of original research and review articles dealing with the structure, distribution, actions and functions of peptides in the central and peripheral nervous systems. The explosion of research activity in this field has led to the identification of over 25 naturally occurring endogenous peptides which affect nervous function and many of which may act as neurotransmitters or neuromodulators. In addition large numbers of peptide analogues have been prepared and there is a growing list of non-peptide compounds which interact with or affect peptidergic systems. Researchers from many disciplines are involved in this field and the new journal will provide a unique opportunity for integration. It is hoped to publish articles on all aspects of the field including chemical, biochemical, anatomical, physiological, pharmacological, behavioural and clinical studies.

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The journal will also publish the monthly bibliography on neuropeptides prepared by the University of Sheffield Biomedical Information Service.

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CYCLIC ADENOSINE MONOPHOSPHATE REGULATES VASOACTIVE INTESTINAL POLYPEPTIDE AND ENKEPHALIN BIOSYNTHESIS IN CULTURED BOVINE CHROMAFFIN CELLS.

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ABSTRACT

When bovine chromaffin cells are dissociated from the adult adrenal gland and placed in culture, they begin to synthesize vasoactive intestinal polypeptide (VIP); they also contain high levels of enkephalin peptides. The regulation of expression of VIP and enkephalin in these cells by cyclic nucleotides was examined. Exposure of cultured chromaffin cells to cholera toxin (CT), forskolin (F), isobutylmethylxanthine (IBMX), 8-bromo-cyclic AMP (8-Br-cAMP) or dibutyryl cyclic AMP (dbcAMP) increases VIP and met-enkephalin biosynthesis as measured by an increase in total (cellular + secreted) VIP and met-enkephalin in treated versus untreated cells. Enkephalin levels increase 1.5 to 3.0 -fold while VIP levels increase 10 to 20 -fold after 48 hours of exposure to the compounds above. Increased enkephalin levels are maximum by 48 hours of exposure; VIP levels are elevated by forskolin or cholera toxin already at 17 hours of exposure. Neither forskolin nor cholera toxin alter catecholamine levels in cultured cells even after 72 hours of exposure. Thus, VIP and ME expression in cultured chromaffin cells can be regulated by intracellular cAMP. Measurement of preproenkephalin messenger RNA ($mRNA^{enk}$) by quantitative Northern blot hybridization with a preproenkephalin complementary DNA probe revealed that exposure of chromaffin cells to forskolin elicits a greater than two-fold increase in $mRNA^{enk}$, suggesting that the regulation of neuropeptide expression by elevated intracellular cAMP occurs at a transcriptional locus.

INTRODUCTION

Cyclic AMP is known to be involved in transduction of secretagogue-stimulated release, and regulation of biosynthesis, of pituitary polypeptide hormones (1-3) and transsynaptic induction of tyrosine hydroxylase in the adrenal medulla and chromaffin cells (4,5). We have recently found that chromaffin cells in culture begin to synthesize VIP, which is absent (or present only in very low levels) in the mature gland in vivo (6,7). These cells also contain enkephalin polypeptides both in vivo and in culture (8,9,10,11). We now report that increasing cAMP levels in chromaffin cells results in induction of both VIP and enkephalin biosynthesis which appears to be transcriptionally regulated. This finding may be relevant to the molecular mechanism of transsynaptic induction of polypeptide hormone biosynthesis not only in the adrenal medulla, but elsewhere in the peripheral nervous system.

MATERIALS AND METHODS

Chromaffin cells were isolated and cultured in 24-well Costar plates as previously described (8). Peptides were extracted, after aspirating the medium from the cells, by addition of 0.1 N HCl, freezing and thawing, sonication, clarification by centrifugation, and vacuum-evaporation of extract aliquots in the glass or plastic tubes used for radioimmunoassay. VIP and ME were measured as described previously (12,13). Drugs were added to the cells in culture (300,000 cells/ml medium or 700,000 cells/1.5 ml medium per well) by removing half of the medium and replacement with an equal volume of medium containing drugs at twice the final concentration. Drugs were added on day 3 of culture (96 hours following plating). Forskolin was added in medium containing dimethylsulfoxide (DMSO) at a final concentration of 0.1%. In some experiments, the vehicle for forskolin was 0.1% ethanol. There was no effect of vehicle (DMSO or ethanol) on the drug effects observed or on control levels of VIP, ME or catecholamines.

For quantitation of $mRNA^{enk}$ cells were washed in ice-cold phosphate-buffered saline (PBS) and incubated for 90 minutes at 42°C in 250 µl per well of SETpK (1% sodium dodecyl sulfate, 10 mM Tris pH 7.6, 5 mM EDTA, 70 µg/ml proteinase K). Incubates were extracted twice with 1 volume of phenol-chloroform (1:1, v/v) to remove protein, and total nucleic acids (TNAs) were precipitated from the aqueous phase in 65% ethanol at -20°C. TNAs were collected by centrifugation and denatured for 5 min at 65°C in 2.2 M formaldehyde/50% formamide containing 20 mM MOPS, 5 mM sodium acetate and 1 mM EDTA at pH 7.0.

Samples were electrophoresed on 1% agarose gels containing 2.2 M formaldehyde, at 150 V for 2-3 hours in 20 mM MOPS/5 mM NaOAc/1 mM EDTA. RNA was electro-eluted ("trans-blotted") onto nitrocellulose paper, which was then baked at 70°C *in vacuo* for 90 min. Filters were pre-hybridized for 2-5 hr at 45°C in 50% formamide, 50 mM $NaPO_4$, 0.1% SDS, 0.1% Ficoll, 0.1% polyvinyl-pyrolidine, 0,1% bovine serum albumin and 5X SSC (750 mM NaCl; 75 mM Na/citrate) containing 250 µg/ml sonicated and denatured salmon sperm DNA and 250 µg/ml yeast tRNA. Filters were hybridized at 42°C in the same buffer containing 10% dextran sulfate and ^{32}P-labeled $cDNA^{enk}$ for 18-24 hours, and washed with 0.2 x SSC/0.1% SDS at 45°C for 20 min. $mRNA^{enk}$ Was quantitated by densitometric scanning of autoradiograms of each Northern blot using an LKB soft laser densitometer.

A recombinant clone of pBR322 containing a DNA insert complementary to bovine $mRNA^{enk}$ (kindly provided by Dr. Peter Seeburg, Genetech Inc., see ref. 14) was grown in the E. coli host MC1061 and harvested without amplification from 500 ml overnight cultures. Plasmid was purified from a cleared lysate by SDS-alkali extraction/cesium chloride centrifugation as described by Maniatis et al. (15). The plasmid was digested with PstI and a 400 base pair fragment within the translated region of $mRNA^{enk}$ was purified by gel electrophoresis and ^{32}P-labeled by nick translation. This probe was used for quantification of $mRNA^{enk}$ as described above.

Total catecholamines were measured using the flucrimetric method of von Euler and Flooding (16).

Peptides were obtained from Peninsula Labs, forskolin from Calbiochem, and cyclic AMP, dbcAMP, IBMX, 8-Br-cAMP and cholera toxin from Sigma.

RESULTS

Forskolin, a reversible and potent stimulator of adenylate cyclase (17), as well as cholera toxin, IBMX, 8-Br-cAMP and dbcAMP, increases total levels of both VIP and met-enkephalin in cultured chromaffin cells following a 48-hour exposure of the cells to these agents (Figure 1). VIP levels increase 10 to 18 -fold due to an elevation both in immunoreactive VIP released from the

Table 1.

TIME COURSE OF FORSKOLIN-INDUCED INCREASE
IN VIP and MET-ENKEPHALIN IN CULTURED CHROMAFFIN CELLS

		VIP		
Time[a]		Cell Content (pg/well)	Medium Content (pg/well)	Total (pg/well)
17	C	33 ± 0.33	56 ± 2.7	89 ± 2.5
	F	597 ± 13***	251 ± 12***	849 ± 17***
24	C	29 ± 2.7	87 ± 3.5	116 ± 1.0
	F	424 ± 29****	818 ± 57***	1242 ± 83***
48	C	55 ± 2.3	75 ± 2.3	130 ± 3.7
	F	769 ± 29***	1002 ± 6.5 ***	1754 ± 46***
72	C	82 ± 5.2	78 ± 6.2	160 ± 10
	F	1183 ± 200**	977 ± 55***	2181 ± 176***

		Met-Enk		
Time[a]		Cell Content (pg/well)	Medium Content (pg/well)	Total (pg/well)
17	C	2267 ± 180	269 ± 13	2535 ± 168
	F	2054 ±106	527 ± 43**	2548 ± 119
24	C	1817 ± 283	206 ± 18	2020 ± 300
	F	1520 ± 106	200 ± 6.5	1720 ± 102
48	C	1978 ± 29	585 ± 18	2563 ± 37
	F	2475 ± 52**	1319 ± 54***	3795 ± 83***
72	C	2143 ± 48	761 ± 12	2904 ± 38
	F	2705 ± 167*	1714 ± 51***	4422 ± 166***

[a]Forskolin (25 μM) was added 96 hours after plating and cells harvested 17,24,48 and 72 hours after addition of drug. C, control; F, forskolin added. 25 μM forskolin increased cyclic AMP levels more than 30-fold, and did not affect spontaneous release of VIP or Met-Enk from the chromaffin cells, following a 30 min exposure (data not shown). Cell catecholamine content was not altered by forskolin at any exposure time. Values are the mean ± S.E.M. of 3 individual determinations (wells). *P< .05, **P< .01, ***P< .001 compared to controls at corresponding times: Student's two-tailed t-test.

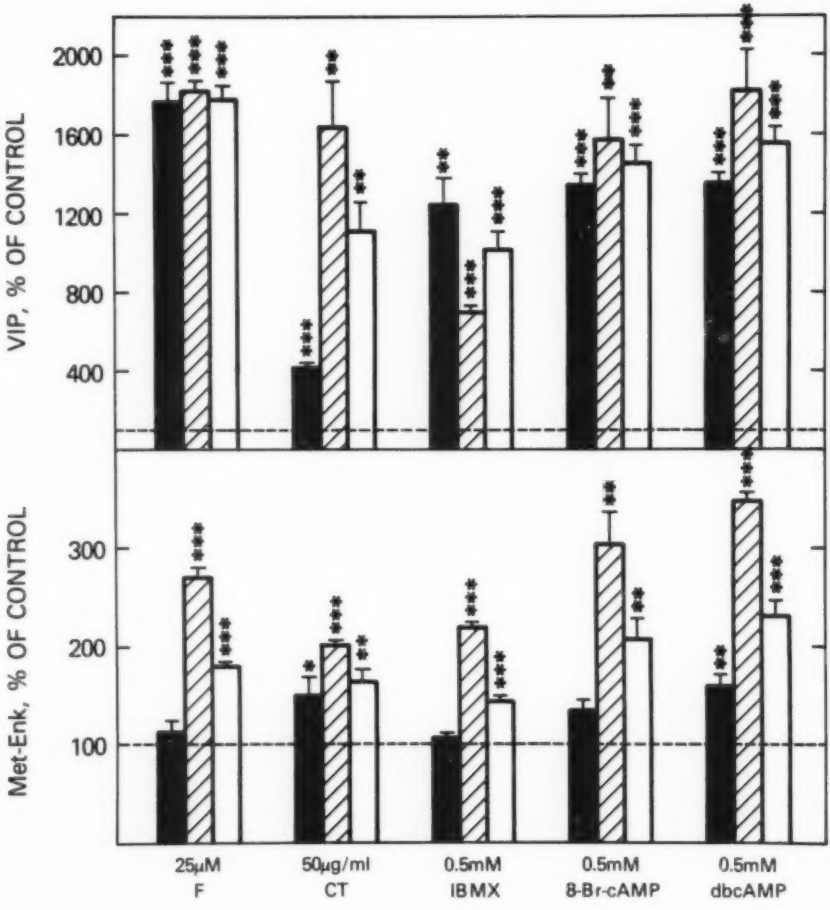

Figure 1

Changes in VIP and met-enkephalin following exposure to agents which mimic or increase intracellular cAMP. ■ cell content; ▨ medium content; □ cell + medium (total) content. Values are the mean ± S.E.M. of 3 separate determinations (wells). Drugs were added to the cultures as described in Methods, 48 hours prior to harvest. *P <.05, **P <.01, ***P <.001, using Student's two-tailed t-test.

cells into the medium and in the cellular content of VIP (Figure 1). The 1.3 to 2.5 -fold induction of met-enkephalin immunoreactivity measured after exposure to forskolin, cholera toxin, IBMX, 8-Br-cAMP and dbcAMP is due mainly to an increase in met-enkephalin released into the medium by the cells (Figure 1).

The time course of VIP and met-enkephalin induction by 25 μM forskolin was examined. 17 Hours after addition of forskolin, VIP levels were elevated 10-fold compared to untreated cultures (Table 1). Both intracellular VIP and VIP released from the cells into the medium were increased. By contrast, forskolin induction of met-enkephalin biosynthesis was apparent only after a 48-hour exposure to the drug (Table 1).

To determine whether the increases in VIP and met-enkephalin biosynthesis elicited by elevation of intracellular cAMP were a consequence of de novo protein synthesis or increased mobilization from a precursor storage pool, cells were treated with 50 μg/ml cholera toxin in the presence of 0.5 μg/ml cycloheximide for 72 hours. Induction of both VIP and met-enkephalin biosynthesis by cholera toxin was abolished by cycloheximide (Table 2).

Table 2

Blockade of Cholera Toxin Induction of
VIP and Met-Enk Biosynthesis by Cycloheximide

	Total (Cell + Medium) Met-Enk (pg/well)		Total Cell + Medium) VIP (pg/well)	
	-CYC	+CYC	-CYC	+CYC
No additions	2563 ± 37	2089 ± 33	130 ± 3.7	58.3 ± 40
Cholera toxin (50 μg/ml)	4180 ± 314	1563 ± 89	1460 ± 189	81 ± 10.2

CYC = cycloheximide, 0.5 μg/ml. Values are the mean ± S.E.M. of three separate determinations (wells). Cells were plated at 300,000/well as in Figure 1 and Table 1.

The mechanism of neuropeptide biosynthesis induction following stimulation of adenylate cyclase was further investigated by measurement of $mRNA^{enk}$ levels in chromaffin cells following a 48-hour exposure to 50 μM forskolin (Figure 2 and Table 3). Total nucleic acids were extracted from chromaffin cell cultures, electrophoresed on agarose gels, blotted onto nitrocellulose paper and submitted to Northern blot hybridization to a ^{32}P-labeled cDNA probe for bovine $mRNA^{enk}$. The autoradiographic signal is localized to a region of the blot corresponding to a molecular size of 1.4 ± 0.5 kilobases, the approximate size of the bovine enkephalin mRNA reported previously (14). This determination was made by ethidium bromide staining of duplicate gels, permitting visualization of the 28S and 18S ribosomal bands as size markers. Densitometric scanning of negative images of UV-trans-illuminated photographs of these gels also permitted relative quantitation of

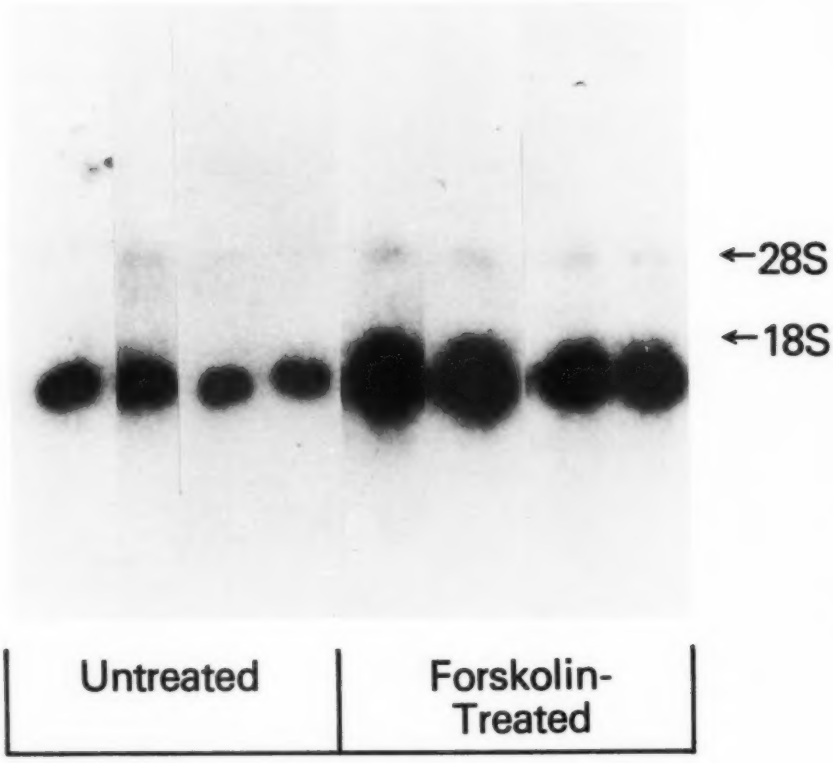

Figure 2
Chromaffin Cell Northern Blots Hybridized with ^{32}P-labeled Enkephalin cDNA. Chromaffin cells in culture (700,000 cells/well) were exposed to 50 μM forskolin or vehicle for 48 hours, beginning on day 3 of culture, and processed for Northern blot hybridization as described in Methods. Each lane represents RNA extracted from about 300,000 cells. The autoradiograms depicted were scanned densitometrically to determine the relative intensity of the autoradiographic signal at the position of migration of $mRNA^{enk}$ in each lane. Linearity of the autoradiographic signal intensity with the size of the absorbance peak upon densitometric scanning was determined by scanning serial dilutions of a pooled forskolin-treated sample.

the amount of RNA applied to the gel for each sample. The amount of total RNA extracted from untreated and forskolin-treated cells was not significantly different, and is about 3 μg of RNA per 300,000 cells.

Exposure to 50 μM forskolin for 48 hours resulted in a 2.3-fold increase in $mRNA^{enk}$ (Figure 2, Table 3). Forskolin elicited a 1.4-fold increase in immunoreactive met-enkephalin in parallel cultures harvested for radioimmunoassay as described in Methods after a 48-hour exposure to the drug (Table 3).

Table 3

	Forskolin-Induced Increase in $mRNA^{enk}$ and Cellular Met-Enk	
	$mRNA^{enk}$	Met-Enk (ng/well)
No additions	18.8 ± 4.0	6.29 ± 0.64
50 μM forskolin	43.0 ± 5.4*	8.97 ± 0.61*

Values represent the mean ± S.E.M of 4 separate determinations (wells). Cells were plated at 700,000/well and exposed to forskolin or vehicle for 48 hours, beginning on day 3 of culture. Values for $mRNA^{enk}$ are arbitrary units, proportional to the area under the peak following soft laser scanning densitometry of the autoradiograms depicted in Figure 2.
*$P < .05$, compared to control (no additions) using Student's two-tailed t-test.

DISCUSSION

Exposure of chromaffin cells in culture to agents which increase intracellular cAMP (forskolin, cholera toxin and IBMX) or mimic its action (8-Br-cAMP and dbcAMP) alters the expression of both VIP and met-enkephalin. There are apparent qualitative differences in the regulation of each neuropeptide by elevation of intracellular cyclic AMP. Thus, forskolin appears to cause a more rapid induction of VIP than of met-enkephalin (Table 1) and induction of met-enkephalin biosynthesis by all agents tested appeared primarily as an increase in secreted enkephalin, whereas both secreted and intracellular VIP were markedly elevated (Figure 1, Table 1). The met-enkephalin antiserum used in this study (RB-4, kindly provided to us by Dr. Steve Sabol, NIH) has only minimal cross-reactivity in our radioimmunoassay (13) for the hepta- and hexapeptides which are the biosynthetic precursors of met-enkephalin, whereas VIP antiserum would be expected to recognize both immature (precursor) and mature (fully processed) VIP (12). Thus, these differences may be a reflection of differential rates of processing of VIP and met-enkephalin from their respective precursors, as well as preferential secretion of mature met-enkephalin pentapeptide from the chromaffin cells.

Cycloheximide blocks cholera toxin induction of both VIP and met-enkephalin biosynthesis (Table 2). This suggests that increased cellular cAMP induces neuropeptide expression at the level of increased protein synthesis, rather than increased peptide precursor processing or decreased neuropeptide metabolism.

Dependence on de novo protein synthesis for induction of neuropeptide biosynthesis implies that induction is a result of increased mRNA translation. This in turn may be a consequence of increased gene transcription, decreased mRNA degradation or increased mRNA translatability. To examine this question, messenger RNA coding for preproenkephalin was quantified by Northern blot hybridization of RNA extracted from untreated and forskolin-treated cells. Forskolin elicited a parallel increase in cellular immunoreactive met-enkephalin and $mRNA^{enk}$ (Figure 2 and Table 3). Similar increases in $mRNA^{enk}$ are elicited by cholera toxin, IBMX, dbcAMP and 8-Br-cAMP (P. Giraud, A. Hotchkiss and L. Eiden, in preparation). These results suggest that the increase in neuropeptide biosynthesis elicited by a rise in intracellular cAMP occurs at the locus of gene transcription, although an effect on $mRNA^{enk}$ stabilization cannot be ruled out by these data. Induction of tyrosine hydroxylase by 8-Br-cAMP in cultured chromaffin cells may also occur via an enhancement of gene transcription (5). Further elucidation of the cellular biochemistry of VIP and enkephalin expression in cultured chromaffin cells may afford some insight into the differentiation of peptidergic phenotype which occurs in the developing nervous system, and the variety of strategies available to neuroendocrine cells in regulating the levels of the neurohormones they produce and secrete.

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MICROHETEROGENEOUS NEUROPHYSINS IN NEWLY FORMED AND AGED NEUROSECRETORY GRANULES

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ABSTRACT

To find out, whether microheterogeneity of neurophysins is due to aging of neurosecretory granules we investigated neurophysin proteins of porcine origin from newly formed and aged granules, as well as from two different extraction methods from whole posterior pituitary glands by high performance liquid chromatography. In newly formed as well as in aged granules all microheterogeneous forms of the neurophysins were present in nearly identical relation suggesting that microheterogeneity is not due to maturation or post matural degradation.

In comparison, the crude material obtained by the method of Uttenthal and Hope showed a comparable content of the different neurophysins while Chauvet's method resulted in a different HPLC pattern. In summary, neurophysin heterogeneity is obviously not due to aging processes.

INTRODUCTION

Neurosecretory granules in the hypothalamo-neurohypophyseal tract are formed in the supraoptic and paraventricular nuclei and transported into the neural lobe. Fractionation of neurosecretory granules from the neural lobe of rat pituitary glands by ultracentrifugation with an isoosmotic gradient resulted in two populations of granules each containing neurophysins, oxytocin and vasopressin. There was evidence that the newly formed granules are osmotically insensitive and that the osmotically sensitive granules (aged) are probably derived from the former, suggesting an aging process within the neurohypophysis (1). In recent studies the presence of microheterogeneous neurophysins in porcine pituitary glands (2) and porcine neurosecretory granules (3) was shown. Similar findings were obtained with human neurophysins (4,5).

Since these microheterogeneous forms are not due to post-mortal degradation in the gland (6) we investigated the content of newly formed and aged granules from porcine posterior lobes in order to see whether they are caused by aging processes.

MATERIAL AND METHODS

Porcine pituitary glands were obtained immediately after death of the animals from the local slaughter house. Neurosecretory granules were prepared according to Scherman and Nordmann (7). 6o posterior lobes were dissected under a microscope and homogenized using a Potter-homogenizer (Braun, Melsungen, W-Germany) in icecold o.3 M sucrose buffered with 1o mM Hepes. After centrifugation at 8oo g for 1o min (4°C) the resulting pellet was homogenized again and centrifuged at 8oo g. Both supernatants were combined and centrifuged at 3 ooo g for 1o min, the resulting supernatant again at 5 ooo g for 1o min. The supernatant was centrifuged at 27 ooo g for 2o min, the resulting pellet (crude granule preparation) was gently stirred in o.3 M sucrose and layered onto continuous isoosmotic sucrose/AG-6227 gradients in cellulose nitrate tubes. In each tube 5.5 ml of o.3 M sucrose and 5.5 ml of o.36 Osm AG-6227 (33 % vol/vol) were pipetted. Centrifugation at 129 ooo g to isopycnic equilibrium was performed for 1 hour. All steps were performed at 4°C.
The resulting neurosecretory granule fractions were either dissolved in double distilled water and lyophilized, or dissolved in double distilled water and dialyzed for 48 hours (4°C, 4 x 5 l aqua bidest., Visking 18/32) or sonified (Branson sonifier) and without any other procedure immediately transferred to HPLC analysis.
For comparison crude neurophysin extracts were prepared according to the methods of Uttenthal and Hope (8) and Chauvet et al. (9). The proteins of the neurosecretory granules were analyzed by sodium dodecyl polyacrylamide gel electrophoresis (1o % SDS).

HPLC experiments were performed with two Altex pumps 11o A and a programmer 42o connected with a variable wave length UV detector (Uvicon 72o, Kontron, Eching, W-Germany). The column was a Aquapore RP 3oo (o.4 i.d. x 25 cm), the flow rate 1 ml/min, detection was performed at 225 nm. A linear gradient from 9o to 3o buffer B was applied in 16o minutes. Buffer B was o.1 M sodium phosphate pH 2.1, the eluent was methanol.

The column was calibrated by separation of neurophysin extracts obtained after the method of Uttenthal and Hope (8) and from granula extracts prepared after the method of Pickup et al. (1o). The neurophysin peaks were collected and characterized by amino acid analysis, polyacrylamide gel electrophoresis pH 8.9, isoelectrofocussing in the range from pH 4 to 5 and N-terminal amino-acid determination thus determining the retention positions of neurophysins I_1, I_2, II_1, II_2 and III. The elution positions of oxytocin and lys-vasopressin were determined with synthetic peptides purchased from Bachem, Burgdorf, Switzerland.

Water was highly purified grade (Millipore Q reagent water system), AG-6227 was purchased from Byk-Gulden, Konstanz, W-Germany, Hepes and sucrose from Sigma, Munich, W-Germany, all other reagents were from Merck, Darmstadt, W-Germany.

RESULTS AND DISCUSSION

Figure 1 compares the chromatograms of the extracts from aged and newly formed granules and of the crude neurophysin extracts after Uttenthal and Hope (8) as well as after Chauvet et al. (9). As shown in figure 2 by sodium dodecyl sulfate gel electrophoresis the aged granules contain nearly no high molecular weight material in comparison to newly formed granules, which is in agreement with the findings of Nordmann and Labouesse in the rat (1) suggesting an aging process of the granule content.

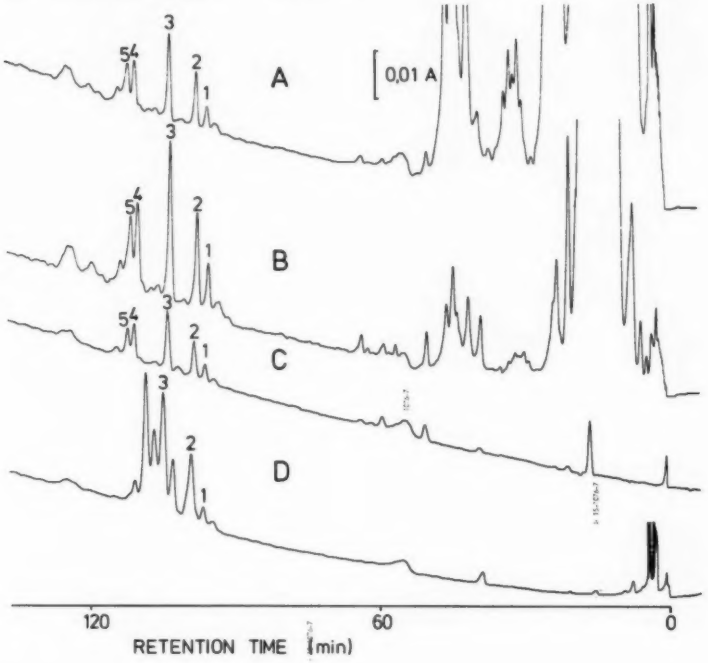

FIGURE 1

Chromatograms of
A) Aged granules (lyzed by sonification).Content of 1 posterior pituitary gland.
B) Newly formed granules (lyzed by sonification). Content of 1 posterior pituitary gland.
C) Extract after Uttenthal and Hope (8).
D) Extract after Chauvet et al. (9).

o.o1 A = o.o1 Absorbance
1 = NP III, 2 = NP I_1, 3 = NP I_2, 4 = NP II_1, 5 = NP II_2

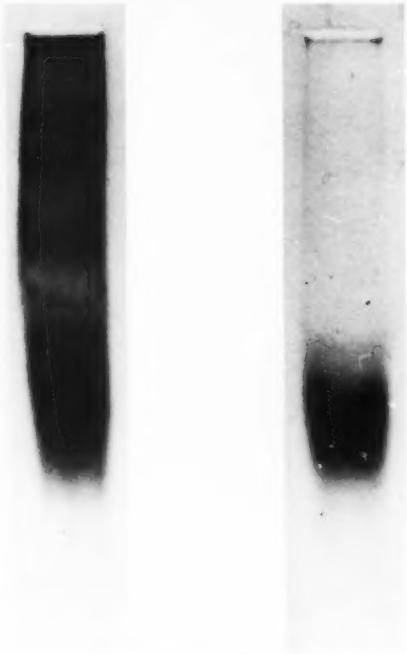

FIGURE 2
SDS gel electrophoresis (1o % SDS) of lyzed newly formed (left) and aged (right) granules (1 mg each).
Stained with Amido black 1o B.

However, the crude neurophysin extract according to Uttenthal and Hope (8) as well as the aged and newly formed granules contain the neurophysins I_1, I_2, II_1, II_2 and III in a comparable relation suggesting that the heterogeneity of neurophysins in the posterior pituitary gland is not due to the aging process. Furthermore, these data suggest that the microheterogeneity is not a result of postmatural degradation. The crude neurophysin extract obtained by the method described by Chauvet et al.(9) contained the neurophysin III, I_1 and I_2 but no neurophysin II. These data show that in granules nearly no material with the retention times of peaks in the Chauvet's crude extract are present suggesting that the extraction procedure concentrates this peak material. These peaks do not increase during the aging process.

Our results show that the microheterogeneous forms are not due to degradation processes during maturation, postmatural, during extraction or postmortal. These findings suggest a possible functional role of these microheterogeneous forms. The importance of microheterogeneity for the biological function has been shown recently, e.g. for apolipoprotein E (11).

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THE EFFECTS OF VASOPRESSIN ON POSITIVELY REWARDED RESPONDING AND ON LOCOMOTOR ACTIVITY IN RATS

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ABSTRACT

It has been suggested that arginine-vasopressin (AVP) enhances cognitive, and especially mnemonic, ability. Most studies have employed shock avoidance paradigms; we report the results of a study in which saline or vasopressin (0, 0.5 or 1 microgram, mcg, per rat, subcutaneous) pre-treated rats learned to press a lever for food reward. AVP was found to have a disruptive effect on aquisition, particularly when the tendency for these rats to produce extreme learning scores was taken into account.

Locomotor activity, with and without vasopressin pre-treatment (0, 0.5, 1 or 2 mcg/rat), was also studied. Only the highest dose significantly reduced activity; therefore, the effects of AVP on acquisition are unlikely to have been caused by motor disruption. The results are discussed in terms of an hypothesis which suggests that AVP enhances arousal, hence influencing performance.

INTRODUCTION

Studies from our laboratory [1-3] and elsewhere [4-6], suggest that AVP and related peptides increase behavioural arousal rather than improve memory and learning processes. This distinction is important; arousal and performance are related by an 'inverted-U' function (the Yerkes-Dodson Law, [7]), and the arousal-enhancement hypothesis suggests that AVP may improve or disrupt performance depending on whether the subject was under- or over-aroused.

Sahgal [1] demonstrated that AVP significantly retards the acquisition of an operant response in congenitally AVP deficient Brattleboro (homozygous diabetes insipidus, HODI) rats. He argued that such a finding is consistent with the arousal hypothesis as HODI rats appear hyper-reactive, and indeed findings suggest that they are anxious, timid and

cautious (Andrews et al, 1983, unpublished, see also [8, 9]). Normal subjects may be not be impaired to the same extent since, as a population, they are not biased towards one extreme of the arousal-performance function. Accordingly, we studied the effects of AVP or saline pre-treatment on the ability of normal rats to acquire lever pressing. This is of particular interest: there have been infrequent reports on the effects of this peptide or its analogues on positively rewarded tasks, but the results have been ambiguous [5, 10-15]. Generally, improved performance following vasopressin administration has been seen when aversive (shock) stimuli have been used [16-18]. The use of operant procedures minimize interference from irrelevant environmental stimuli, which may have contributed to the conflicting results obtained in some previous appetitively rewarded studies.

One further point deserves consideration. We have found, in passive avoidance studies, that bimodal performance scores are often observed after AVP administration. These extreme scores would be more likely to occur if the arousal-performance curve was skewed, rather than symmetrical. An arousal enhancing substance might then improve performance only slightly for subjects at a below optimal state of arousal (shallow ascending limb of the curve), but disrupt performance relatively severely for initially highly aroused subjects (steeper descending limb). In this study, the possibility of a bimodal performance score distribution was considered.

EXPERIMENT 1: EFFECTS OF AVP ON AUTOSHAPING

METHOD

Subjects

These were 57 female Norwegian Hooded rats (Bantin & Kingman Ltd., Hull, U.K.), weighing 150-180g and individually housed under diurnal conditions with free access to water, but on a 23 hr food deprivation schedule.

Apparatus

The equipment consisted of 4 Skinner boxes (Campden Instruments Ltd., London, U.K.), fitted with retractable levers, and controlled by microcomputer (Acorn Computers Ltd., Cambridge, U.K.), using ONLIBASIC [19] software.

Procedure

On the first day of training, 20 food pellets were placed in the magazine tray, the magazine light switched permanently on, covering flap open and levers retracted. Each rat was placed in the box for 5 mins and then removed.

On the second day the rat was again placed in the box for 5 mins but this time the randomly activated pellet dispenser provided occasional food. On the third habituation day, the rat was allowed 15 mins in the box; pellets were delivered on a random schedule at a rate of about 1 per min, and the computer recorded all entries into the food tray, ie magazine responses. Only animals which entered the food magazine and ate the pellets were used in the learning trials; it is reasonable to assume that an animal which has not at this time learnt to associate the magazine tray with food, will not associate food delivered to the magazine with the appearance/disappearance of the levers.

Day 4 was the first acquisition day, and rats were divided into three groups: two drug and one control (N = 19 each group). Each animal received a subcutaneous (s.c., 0.5 ml, neck) injection of either saline or AVP (Cambridge Research Biochemicals, Cambridge, U.K.; 0.5 or 1 mcg in 0.5 ml saline) immediately before each day's testing. These doses were chosen after a careful search of the literature; they have been reported to enhance performance. A learning trial began with the illumination of the house light and the emergence of one lever (equi-probable, left or right). After 30 sec the lever was retracted, a pellet delivered and the magazine light switched on. If the rat made a lever press within this time, the lever was immediately withdrawn and food delivered. This procedure is similar to 'autoshaping' protocols described for pigeons [20] and rats [21]. Fifty acquisition trials per day were given, and training stopped when 5 consecutive responses were made or three days training (150 trials) had occurred. Five responses were regarded as the learning criterion; rats can activate a lever by accident, and therefore a single response may not be indicative of learning.

Twenty-four hrs following acquisition, the rat was allowed to respond (50 trials) on the levers for food reward in order to verify that lever pressing was retained. Saline and AVP injections continued as before.

Data were analysed using the Kruskal-Wallis [22] and information statistic [23] tests. This latter test, which is a non-parametric equivalent of analysis of variance, is particularly suited to the analysis of such data (see [2]).

RESULTS

Originally, it seemed possible that the number of magazine entries during habituation was inversely related to learning scores, and thus they might be of some use in predicting performance following AVP. Although there was a trend in this direction, it was not significant. The scattergrams, and lines of best fit, are shown in Fig. 1.

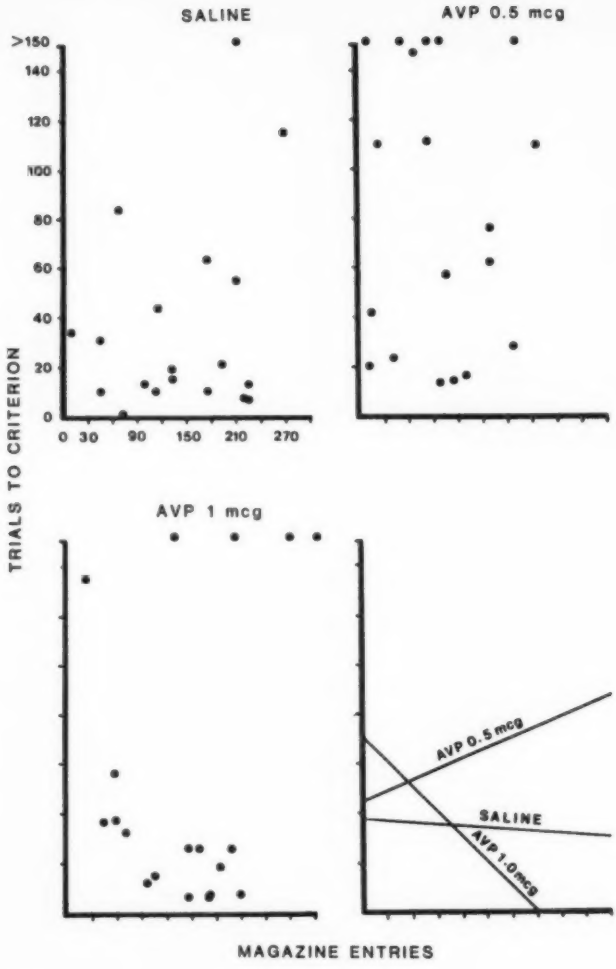

FIGURE 1: Trials to acquisition in the lever pressing task plotted against number of magazine entries during the session prior to training. Best fit lines were drawn by minimizing the square perpendicular distances to the line itself, ie error variances were assumed to be equal for both (X,Y axis) variables (see [24]). None of the correlation co-efficients were significant. Therefore, scores along the X-axis (magazine entries) can be collapsed, but are shown here for illustrative purposes.

The Kruskal-Wallis test just failed to show an overall significant difference between the acquisition scores of the three groups ($H = 5.95$ $df = 2$ $p > 0.05$; critical value for $H = 5.99$). However, inspection of the data (Fig. 1) indicated a tendency towards extremes in the acquisition scores of the drug-treated animals, a factor which would be obscured by a measure of purely central tendency such as the Kruskal-Wallis test. Accordingly, the data were re-analysed using the information statistic: the data were cast into three drug groups (0, 0.5 and 1 mcg AVP) and 3 acquisition score categories (< 50, 50 - 100, > 100 trials to criterion, categories I - III respectively).

This analysis revealed a significant overall difference ($2\hat{I} = 8.91$, $df = 4$, $p < 0.05$, 1-tailed), indicating that AVP had an effect on acquisition. However, there was no significant difference between the two doses of AVP used ($2\hat{I} = 4.03$, $df = 2$, NS), and the data were therefore collapsed into one drug category and compared with the control group (see Table 1). A test for bimodal effects was carried out by comparing the middle and extreme categories. Despite the greater tendency to extreme scores in the drug-treated animals, this test was not upheld (category I vs II: $2\hat{I} = 0.01$, $df = 1$; category II vs III: $2\hat{I} = 2.47$, $df = 1$, both NS).

Trials to Criterion:	< 50	50 - 100	> 100
Saline	14	3	2
AVP	20	4	14

TABLE 1: Learning scores of AVP- and saline-treated rats divided into categories for analysis by the information statistic. Note that the two AVP doses have been collapsed, since they were not significantly different from each other.

DISCUSSION

These findings do not support the hypothesis that AVP has beneficial effects on cognition, including appetitive learning processes [15]. However, inspection of the raw data showed large individual differences in the learning scores. Thus, some AVP-treated animals appeared to learn quickly and others relatively slowly or not at all; the Kruskal-Wallis test will average out these differences and obscure any specific effects of AVP on individual animals.

When the data were re-analyzed in order to take account of the tendency for extreme scores in the data, AVP was found to have a significant deleterious effect, although the data were not significantly bimodal in nature. The observed pattern of scores following AVP treatment (see Table 1), does not support hypotheses which suggest either a uniform enhancement, or disruption, of learning and memory processes. Rather, the data spread suggests some motivational effect, and indeed this is in line with our hypothesis that AVP acts on arousal. This latter theory would predict a deleterious effect on the performance of only some, initially highly aroused, animals, instead of the uniform improvement or disruption of performance implicit in the former hypotheses.

Some theorists have recently suggested that anxiety as well as arousal increase in response to stimulation [25], such that after a certain point the negative effect of anxiety on performance is greater than the beneficial effects of increased arousal; the net effect would be for arousal and performance to relate in an inverted-U manner [7, 25, 26]. Perusal of the raw data suggested that the number of entries made into the food magazine during habituation might be useful as a measure of arousal/anxiety, and thus be used to predict each animal's response to AVP. Anxious animals might be expected to make fewer entries into the food tray, and be more disrupted by an arousal enhancing agent such as AVP. Despite a suggestion that high numbers of magazine entries and low acquisition scores were related, the correlation fails to reach an acceptable level of significance. However, it may be that an independent measure of anxiety/arousal might be found in the response of naive animals to a novel environment, and would therefore be a useful aid in predicting the effects of an arousing agent on the performance of individual animals.

In this context the work of Alliot and Alexinsky [10] is especially relevant: while studying the effects of lysine vasopressin (LVP) on the learning of a light-dark discrimination task, they initially saw no effect of LVP treatment. However, the number of bar presses decreased, and, a differential effect was found when the LVP and saline groups were divided into "good" and "bad" learners based on performance scores from a previous task. The inverted-U arousal hypothesis offers an attractive explanation for these divergent scores. In a heterogenous population of animals as used by Alliot and Alexinsky and ourselves, we would expect the initial arousal states to be spread around the optimal portion of the (skewed) arousal-performance curve. The effects of an arousing substance would be to separate the subjects into two groups where performance may be improved if a given subject's

arousal was initially low, or impaired if it was high.

EXPERIMENT 2: EFFECTS OF AVP ON LOCOMOTOR ACTIVITY

It can be argued that the doses of AVP used in the previous learning experiment may have caused a non-specific motor suppression, which might interfere with acquisition of lever pressing. This possibility was tested by assessing locomotor activity after pre-treatment with saline or AVP at different doses, including a relatively high dose.

METHOD

Subjects

These were 8 saline treated animals from the previous experiment; several days were allowed to lapse during which food and water were available ad libitum.

Apparatus

The test chambers were eight black Perspex activity cages, measuring 40 x 20 x 20 cm, provided with a metal mesh floor. Each cage was fitted with 2 red light photocells (20 cm apart, and 4 cm above the floor), connected to an Acorn microcomputer.

Procedure

The rats were habituated in their activity cages for 90 mins per day for one week. Following this period, injections of either saline (0.5 ml s.c., neck) or AVP at one of three doses (0.5, 1, or 2 mcg in 0.5 ml saline) were given immediately before subsequent testing. At least 3 days were allowed between AVP doses, and each dose was tested twice. The protocol was a 'reverse block' design, the peptide (mcg/rat) being administered and data collected in this order: saline, 1, 2, 0.5, 0.5, 2, 1, saline. Thus, each subject yielded 8 data days, 2 for each dose; data from each pair of identical doses were subsequently averaged in an effort to minimize order effects.

The computer recorded (a) counts per photocell over 18 successive 5 min time bins, and (b) all counts with a duration of less than 0.2 sec, ie very brief interruptions (VBI's), which represent behaviours such as grooming, shaking and rearing [2]. Data were analysed by a 2-factor (dose, time) repeated measures ANOVA, and a posteriori comparisons by the Neuman-Keuls procedure [27].

RESULTS

Statistical analysis of the total counts data indicated that both main effects (dose, time), and their interaction were significant: $F(3,21) = 4.86$, $p < 0.01$; $F(17,119) = 14.7$, $p < 0.01$; $F(51,357) = 3.03$ $p < 0.01$. Post-hoc comparisons showed that only the highest dose (2 mcg) of AVP significantly depressed overall activity. Similar analysis of the VBI data revealed only the time main effect to be significant: $F(17,119) = 5.25$, $p < 0.01$.

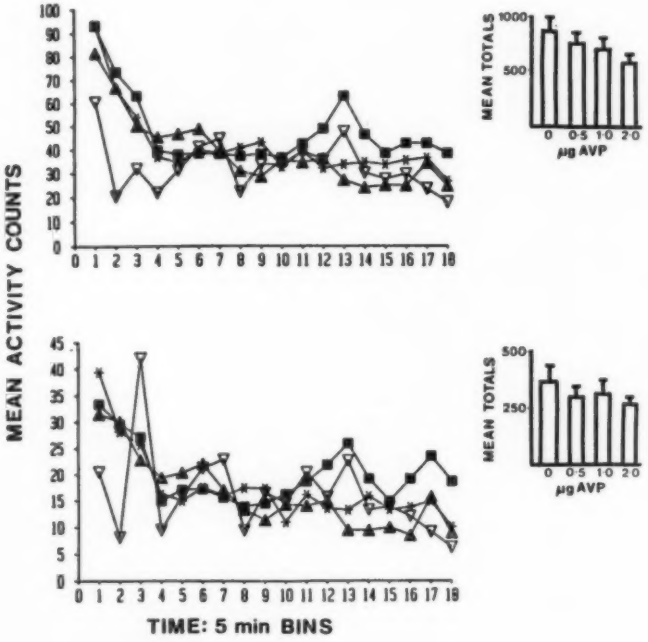

FIGURE 2: Total mean activity counts (top), and VBI's (bottom) of rats treated with AVP. Key: ■ = saline; * = 0.5; ▲ = 1; ▽ = 2 mcg AVP. Histograms show total response (+/- SEM) over the 90 min test period. Only 2 mcg of AVP significantly depressed overall activity.

DISCUSSION

These results indicate that AVP can suppress locomotor activity; inspection of Fig. 2 (top) suggests that this is dose dependent. Ettenberg et al [28] have also reported a

dose dependent inhibition of locomotor activity in AVP treated rats. However, this was apparent for two doses of AVP lower than those used here. It is not immediately apparent why there should be this discrepency, but it could be attributed to sex and strain differences. This suppression of activity is also the opposite of that obtained in our earlier study [1], using HODI rats, and serves to emphasize strain differences. However, we have noticed that a high dose of AVP intitially depresses activity during the first 20 min in both HODI as well as the normal rats studied here.

We have suggested that the disruptive effect of AVP on the acquisition of lever pressing in HODI rats may have been due to this initial inhibitory effect on motor ability [1]. As neither dose (0.5 or 1 mcg) of AVP used in the acquisition task had any significant effect on locomotor activity in this study, it seems unlikely that AVP affects acquisition by non-specifically suppressing motor behaviour.

Inspection of the VBI data (Fig. 2, bottom), shows that 2 mcg of AVP depresses this activity during the first 10 minutes and is followed by a large but short lived increase. This correlates well with gross observations of high AVP doses on normal rats: shortly after injection there is a rigid ataxia, followed 10 - 15 minutes later by increased grooming and consequently little motor activity. These observations are well reflected in the figure.

GENERAL DISCUSSION

The first experiment indicated that AVP (0.5 and 1 mcg, s.c.) had a disruptive effect on the acquisition of a food rewarded lever pressing response in normal female rats. AVP-treated rats tended to yield extreme high and low scores; post hoc analysis designed to take this data spread into account showed that although the disruption was significant, the scores were not significantly bimodal in nature. Identical doses of AVP to those used in the first study had no effect on locomotor activity, suggesting that learning scores were not confounded by non-specific motor suppression. A higher dose (2 mcg) was required to significantly suppress activity.

This study adds further support to the increasing evidence that AVP may affect performance by acting on arousal rather than other cognitive processes [1-6]. One difficulty with this hypothesis is the lack of specificity in predicting the exact response of individual animals to an arousal enhancing agent. Clearly, some measure of a subject's arousal level is required so that an enhancement or disruption of performance under the influence of such

drugs can be predicted. In the present study, the use of magazine entries during habituation as a broad measure of anxiety, did not produce a correlation with aquisition scores of the type necessary to fulfil this role. However, the future availability of such measures must be seen as a useful adjunct to the development of the AVP-arousal hypothesis.

It is well known that systemically administered AVP can cause unpleasant states within rats, and it could be argued that the disruption in performance was due to this aversive component of AVP administration prior to testing. However, we have previously shown that AVP does not affect responding on a lever pressing task once learnt [1]. In addition, doses which affect performance on this task do not significantly interfere with locomotor activity, which noxious stimuli often do; moreover, the fact that AVP disrupts only some animals argues against this as the mechanism of action in this case.

It may be supposed that arousal would depend on current internal and external factors, and might therefore change fairly rapidly from moment to moment. However, there is good reason to believe that certain neural mechanisms constantly monitor arousal level, and prevent transient fluctuations [29]. Arousal is therefore kept at a broadly stable level, although the actual magnitude depends on the individual subject's reactivity. In the absence of any marked change, the animal's arousal level would remain fairly steady, although it can be expected to decrease slightly with increasing familiarity (habituation). This hypothesis gains support from our earlier study using HODI rats [1], where AVP disrupted learning in the relatively unfamiliar Skinner box, presumably by inducing over-arousal (or high anxiety) in all subjects. On the other hand, locomotor activity was not suppressed in the well-habituated (ie familiar) test cages. The work of Brito and his co-workers [8, 9] provides additional support for this hypothesis. They have shown that HODI rats are more 'timid', and less active than normal animals in a novel environment, a difference that tended to disappear as the animals habituated to the test environment.

The AVP arousal hypothesis would predict that AVP would exaggerate this hyper-reactivity, and adequately explains the greater level of disruption seen in the performance of nearly all the HODI rats tested in an identical procedure [1], as opposed to only some of the animals used here. This also adds further weight to our suggestion that some measure of underlying arousal/anxiety levels would be useful in future research.

A comparison of the predictions and implications of

our inverted-U arousal hypothesis with traditional cognitive-mnemonic hypotheses is of interest. A substance (eg AVP) which is supposed to directly enhance learning or memory should not cause their disruption under any circumstances. In fact, to have any value as a promoter of cognitive function, AVP should improve performance regardless of the nature of the task or the reinforcer used. In common with other studies, our results [1-3] show that disruption is frequently observed in at least a proportion of subjects, especially when initial selection has not excluded relatively poor (high anxiety?) performers (see ref. 10). While these results weaken the case for cognitive enhancement, the inverted-U arousal hypothesis is uniquely capable of encompassing these contradictory, and seemingly difficult to interpret observations.

We conclude that the effect of AVP on performance depends on the idiosyncratic behavioural arousal (or anxiety) state. Emphasis is thereby placed on performance (arousal) rather than on learning variables, and this has crucial theoretical, empirical and clinical implications.

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INTRACAMERAL ADMINISTRATION OF NEUROTENSIN INDUCES MIOSIS IN THE RABBIT

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ABSTRACT

The present investigation was designed to evaluate the effect of selected peptides on pupillary diameter and intraocular pressure (IOP) in rabbits.

Intracameral (IC) administration of neurotensin (NT) in doses of 5-100 µg produced a significant, long lasting and dose-dependent decrease in pupillary diameter without affecting IOP. NT-induced miosis appears to be relatively specific because a variety of peptides including Gn-RH, somatostatin, met-enkephalin, bombesin, leu-enkephalin or NT_{1-6}, (a biologically inactive N-terminal fragment of NT), produced no effect on pupillary diameter; only substance P produced miosis similar to NT when tested in a dose equimolar to 30 µg of NT. In addition, peripheral (intravenous) administration of NT (100 µg/kg) was equally ineffective.

Inhibition of prostaglandin synthesis with indomethacin, did not prevent subsequent NT-induced miosis. Finally, IC administration of an effective dose of NT (30 µg) did not alter the protein concentration in the aqueous humor.

These results indicate that NT-induced miosis is not mediated by endogenous prostaglandins and that this effect of NT does not appear to involve disruption of the blood-aqueous barrier, suggesting that NT may play a role in regulation of pupillary diameter

INTRODUCTION

It has become increasingly evident in recent years that peptides, which are heterogeneously distributed throughout the central nervous system (CNS) and in endocrine cells of peripheral organs (e.g., the gastrointestinal tract) play an important role in neurotransmission. This view is based on data provided by neuroanatomical, electrophysiological and biochemical studies. Thus many of these peptides have been localized in cells and fibers in the CNS, have been

demonstrated to alter the rate of neuronal firing after microiontophoretic application, and mechanisms for biosynthesis and degradation of peptides have been identified in nervous tissue (1,2). Recent radioimmunological and immunocytochemical evidence has revealed the presence of many peptides in the visual system (3,4,5).

The ocular peptides thus far identified include substance P (6), vasoactive-intestinal peptide (VIP, 7), thyrotropin-releasing hormone (TRH, 8), somatostatin (SRIF, 9), met-and leu-enkephalin (10,11), glucagon (4), gonadotropin-releasing hormone (Gn-RH, 4), neurotensin (NT, 12), α-melanocyte-stimulating hormone (α-MSH, 13), and ACTH (4). With the exception of substance P which has been demonstrated to produce a marked intraocular hypertension, miosis and leakage of intravascularly injected dye and protein into the aqueous humor after intracameral injection (14,15), very little is known concerning the functional significance of these peptides in ocular tissue. Accordingly, the present investigation was designed to evaluate the effect of some of these peptides on pupillary diameter and intraocular pressure in the rabbit.

MATERIALS AND METHODS

Adult albino New Zealand rabbits (3-5 kg) from Franklin Laboratories (Wake Forest, NC) were housed in a controlled environment animal facility and fed laboratory chow and water ad libitum. Rabbits were habituated to the animal facility for one week prior to experimental use. Animals were anesthetized with intramuscular ketamine hydrochloride (35 mg/kg) and xylazine (5 mg/kg). Pupil diameter was measured with a Thorpe Caliper (V. Mueller, Chicago, IL) and intraocular pressure (IOP) with an Alcon Applanation Pneumatonograph (Fort Worth, TX) in both eyes at 5-min intervals for 15 min prior to drug or peptide administration, and then at 5 min intervals for one hour post-injection. Intracameral injection of peptides or vehicle (10 µl of 0.9% NaCl) was performed at the limbus of the right eye by means of a 50µl Hamilton syringe with a 30 gauge needle.

In the first set of experiments, all peptides were initially injected intracamerally (IC) in a dose equimolar to 30 µg of neurotensin (NT). These included: substance P, leu-enkephalin, met-enkephalin, Gn-RH, SRIF, bombesin and NT_{1-6}, a biologically inactive N-terminal fragment of NT (16).

In the second series of experiments, several doses of NT (5, 10, 30, 60 and 100 µg) were given IC. Pupil diameter and IOP were measured as described above.

In a third series of experiments, NT (100 µg/kg) was injected intravenously (IV). This dose of NT has been previously reported to produce peripheral endocrine and vascular effects (17,18).

Because preliminary studies revealed that IC NT (30 µg) produced a significant unilateral miosis without affecting IOP, this dose of NT was chosen for subsequent experiments. To determine whether the effect of IC NT on pupillary diameter is mediated by endogenous prostaglandins, indomethacin (5 mg/kg), an inhibitor of prostaglandin synthesis (19), was administered subcutaneously 30 min before IC NT (30 µg). This dose of indomethacin has been previously reported to inhibit prostaglandin synthesis in both the CNS and in peripheral organs (20,21).

In a final set of experiments, groups of rabbits (n = 4/time point) were injected IC with vehicle (10 µl 0.9% NaCl) or NT (30 µg). 0, 15 and 30 min post injection, a sample of aqueous humor (200 µl) was slowly aspirated from the anterior chamber of both eyes with a 30 gauge needle, evacuating it to the point of collapse but avoiding direct trauma to the iris or lens. The protein concentration in all aqueous humor specimens was determined by the method of Lowry et al. (22).

Neurotensin, substance P, leu-enkephalin, met-enkephalin, Gn-RH, bombesin and SRIF were obtained from Bachem (Torrance, CA) NT_{1-6} was purchased from Cambridge Research Biochemicals (Harston, Cambridgeshire, U.K.). Indomethacin (Sigma Chemical Co., St. Louis, MO) was dissolved in 5% sodium bicarbonate and adjusted to pH 8.4 with 1N NaOH.

Data were analyzed with one-way ANOVA followed by Dunnett's test for multiple comparisons or Student's t-test for paired samples. The dose-response curve was generated with the least-square method followed by simple linear regression analysis. In all statistical tests, P values of 0.05 or less were considered to represent significant differences between treatment groups.

RESULTS

The results of the first series of experiments are presented in Table I.

Table I. Effect of intracamerally (IC) administered neuropeptides on pupillary diameter (PD) and intraocular pressure (IOP) in rabbits. All substances were injected IC (into the right eye) in doses equimolar to 30 µg of neurotensin, and IOP and PD were measured at 5 min intervals for 60 min. The results are expressed as the averaged response for PD or IOP over the 1 hr period.

Treatment	N	PD (mm ± S.E.M.)		IOP (mm Hg ± S.E.M.)	
		Right Eye	Left Eye	Right Eye	Left Eye
Saline	12	6.9 ± 0.2	7.0 ± .0.1	18.7 ± 1.3	15.2 ± 1.1
Neurotensin	12	4.1 ± 0.2**	7.3 ± 0.1	18.0 ± 1.2	15.5 ± 1.5
Substance P	4	3.0 ± 0.3**	6.9 ± 0.4	24.1 ± 0.9**	16.6 ± 0.6
Somatostatin	4	7.6 ± 0.2	7.8 ± 0.2	15.0 ± 1.1	13.6 ± 0.8
Leu-enkephalin	4	7.3 ± 0.1	6.9 ± 0.2	19.4 ± 1.6	15.4 ± 0.6
Met-enkephalin	4	7.2 ± 0.5	7.1 ± 0.4	16.8 ± 1.0	14.1 ± 0.9
Gn-RH	4	7.3 ± 0.3	7.2 ± 0.2	20.8 ± 1.7	19.4 ± 0.8
Bombesin	4	6.0 ± 0.1	6.8 ± 0.3	15.2 ± 0.3	14.0 ± 1.0
NT_{1-6}	4	7.4 ± 0.1	6.7 ± 0.2	20.0 ± 1.5	18.8 ± 0.7

** $p < 0.01$ when compared to saline-treated controls (Dunnett's test for multiple comparisons).

As previously reported (14), IC administration of substance P produced a significant ($p<0.01$) miosis and a concomitant increase in IOP when given in a dose equimolar to 30 µg of NT. Neurotensin (30 µg) also produced a significant ($p<0.01$) decrease in pupil diameter but, unlike substance P, did not affect IOP. The effects of both substance P and NT appeared to be relatively specific because a number of other peptides tested in this study in a dose equimolar to 30µg of IC NT did not significantly influence IOP or pupil diameter. These included SRIF, leu- and met-enkephalin, bombesin, and Gn-RH. In addition, NT_{1-6} an N-terminal fragment of NT was equally ineffective in this paradigm. This hitherto undescribed effect of intracamerally administered NT was shown to have a rapid onset (~ 5 min) and a prolonged duration (Fig. 1A). The miosis induced by IC NT was restricted to the injected eye. Simultaneous recording of IOP (Fig. 1B) confirmed observations made in the first set of experiments; IC NT did not alter IOP over the 1 hr period. In contrast to the clear miotic effect of IC NT (30 µg), I.V. neurotensin (100µg/kg) was devoid of this property (data not shown). Dose-response relationships for NT-induced miosis were examined next (Fig. 2); IC NT (5-100µg) produced a significant ($p<0.01$) and linearly dose-dependent reduction of pupil diameter. None of the different doses of IC NT tested had any effect on IOP (data not shown).

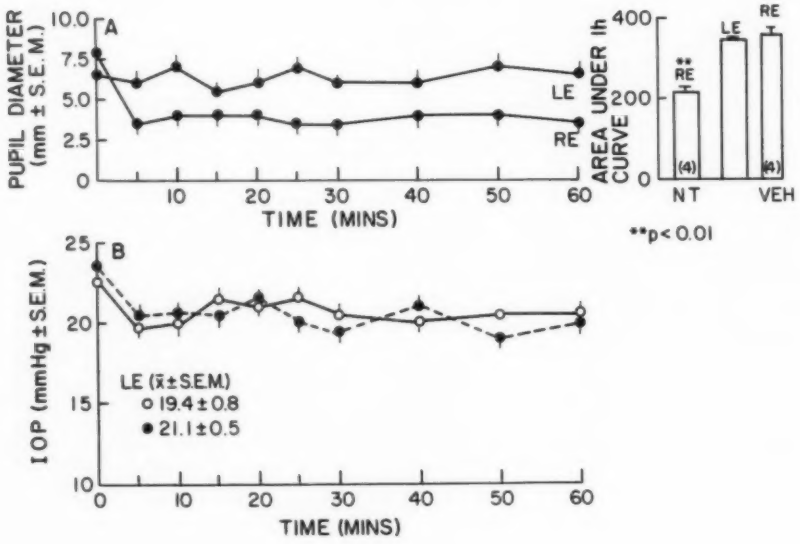

Figure 1

Effect of unilateral intracameral administration of neurotensin (30µg) or vehicle (10µl 0.9% NaCl) on pupil diameter (1A) or IOP (1B). Neurotensin produced significant miosis without affecting IOP (m = 4 rabbits/group). Neurotensin (•), vehicle (o).

**$p<0.01$ vs. left eye (LE) or right eye (RE) of saline-treated controls (Dunnett's test).

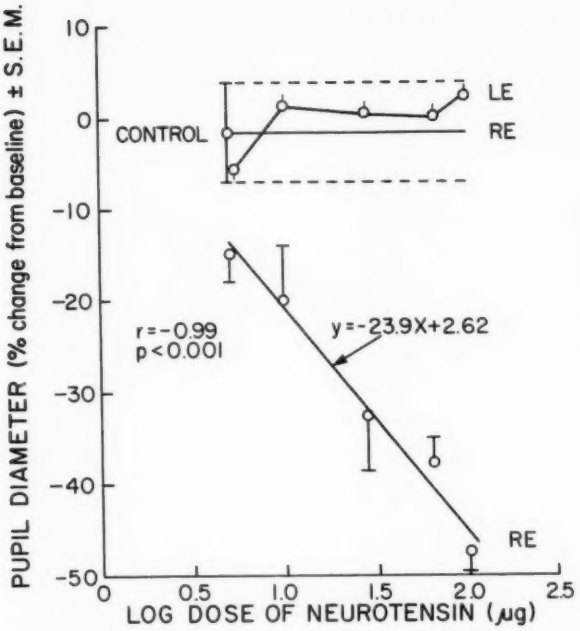

Figure 2: Dose-response relationship for neurotensin-induced miosis. Several doses of neurotensin (5-100μg) were injected intracamerally and pupillary diameter determined as described above (see methods for details). Neurotensin-induced miosis was linearly dose-dependent with a signicant ($p<0.01$) correlation coefficient (r). Note that these doses of neurotensin did not affect pupil diameter in the LE.

Additional studies were undertaken to investigate possible mechanisms by which NT produced miosis. Intracameral administration of NT in a dose already shown to produce significant miosis (30μg) did not affect protein concentration in the aqueous humor 15 or 30 min post-injection (Fig. 3), a time at which IC NT had produced miosis. Finally, indomethacin pretreatment in a dose previously reported to inhibit prostaglandin synthesis (19,20) did not antagonize NT-induced miosis (data not shown).

DISCUSSION

This report describes a hitherto unknown effect of intracameral administration of the endogenous tridecapeptide NT in rabbits i.e., a reduction of pupillary diameter without affecting intraocular pressure. This miosis was shown to be relatively specific because only substance P, as previously reported (14), produced significant miosis. Unlike NT this was associated with a concomitant

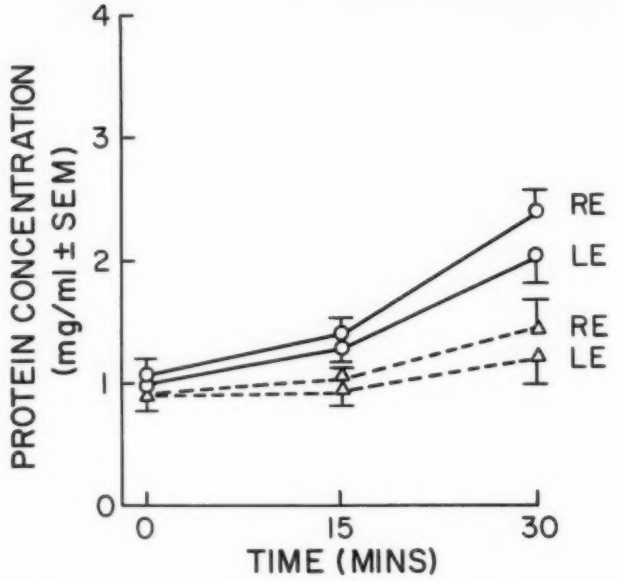

Figure 3: Effect of unilateral intracameral injection of neurotensin (Δ) or vehicle (o) on protein concentration in the aqueous humor. Groups of rabbits (n=4) were injected into the anterior chamber of the RE with neurotensin (30μg) or vehicle (10μl of 0.9% NaCl) and the protein content of both eyes was determined at 0, 15 or 30 min after injection.

increase in IOP. In contrast, a number of other peptides did not have any effect on IOP or pupil diameter when tested in a dose equimolar to 30μg of NT. These included: SRIF, Gn-RH, NT_{1-6}, bombesin, and the opioid peptides, leu- and met-enkephalin.

The null effect of bombesin is particularly interesting because it has been shown that bombesin shares with NT the ability to exert profound effects on thermoregulation, glucose metabolism, food consumption, and pituitary hormone secretion after intracisternal or intracerebroventricular administration in rats and mice (23).

The null effect of met- and leu-enkephalin on pupillary diameter is in contrast to the previously described mydriatic effect of morphine and enkephalins in mice (24). The lack of effect of the enkephalins on pupillary diameter might be attributable to a number of factors such as species differences, dose, route of administration and differences in biological stability of the enkephalins. However, Korczyn et al. (24) used D-ala-D-leu-enkephalin, a protected structural analog of the naturally-occurring leu-enkephalin pentapeptide.

The lack of effect of NT_{1-6} indicates that NT-induced miosis is specific to a portion of the NT molecule and resonate with results obtained in previous studies (16). The lack of effect of NT_{1-6}, SRIF, MET- and leu-enkephalin and Gn-RH on pupillary diameter supports the contention that the ability of NT to induce significant miosis is not merely a property inherent to all peptides.

Because intracameral NT produced miosis, additional studies of dose, route specificity, and putative mechanisms of action were undertaken. Intracameral injection of NT (5-100µg) produced a dose-dependent miosis (ED_{50} = 25.2µg); this dose closely resembles the ED_{50} for other effects of NT (i.e., NT-induced gastric cytoprotection) (16,25). Two findings are of particular interest. First even high doses of IC NT (60-100µg) did not affect IOP. In addition, NT-induced miosis is restricted to the injected eye, thereby, excluding the possible interophthalmic passage of the peptide to the opposite eye. This latter observation is of importance because the consensual reaction to ocular trauma which results in subsequent elevation of IOP, miosis, and accumulation of aqueous protein in the contralateral eye, appears to be mediated by humoral factors transported through the interophthalmic communicating artery (26). We also found that a large IV dose of NT (100µg/kg) which has been demonstrated to produce clear peripheral vascular and endocrine effects (17,18) did not induce miosis. These results suggest that NT-induced miosis is not mediated by the systemic hypotension or marked hyperglycemia produced by IV NT.

From a pathophysiological point of view, the anterior segment of the rabbit eye responds characteristically to mechanical or chemical irritation with miosis, vasodilatation, and disruption of the blood-aqueous barrier (26). This allows the IOP to rise and proteins to enter the aqueous humor. Prostaglandins, the differential reactivity of ANS, and axonic reflexes of primary sensory fibers have all been implicated in this locally-mediated inflammatory reaction of the eye to trauma (28,29).

As noted above certain of the mechanisms that might serve as potential intermediates in NT-induced miosis were investigated. Neurotensin, in a dose sufficient to produce miosis did not affect protein concentration in the aqueous humor. Moreover, blockade of prostaglandin synthesis with indomethacin did not prevent subsequent NT-induced miosis. These findings taken together indicate that NT-induced miosis is probably not secondary to the activation of mechanisms previously demonstrated to play a critical role in the acute development of ocular inflammatory reactions.

In conclusion, the results furnished in the present report are not inconsistent with the hypothesis that endogenous peptides may play an important role in regulation of pupillary diameter.

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ANALYSIS OF BINDING SURFACES: A METHODOLOGY APPROPRIATE FOR THE INVESTIGATION OF COMPLEX RECEPTOR MECHANISMS AND MULTIPLE NEUROTRANSMITTER RECEPTORS

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ABSTRACT

In this study we describe a novel experimental design: analysis of three-dimensional binding surfaces. Evidence is presented that a binding surface provides greater information content than does a single binding isotherm. This approach to the design and analysis of ligand binding studies should facilitate quantitative studies of the opiate receptor.

INTRODUCTION

An underlying principle of scientific investigations is that the question asked determines the methodology used to answer the question. A methodology appropriate for questions about multiple neurotransmitter receptors (1,2) is computer-assisted data analysis. In a typical study, an unlabeled drug is used to displace the binding of a radiolabeled ligand. This single displacement isotherm is then analyzed by a nonlinear least squares curve fitting progrm for the values of the parameters of the hypothesized model.

As recently pointed out by Rothman et al. (3), the observed binding is a function of the ligand and inhibitor concentration,

$$B = F(L, I)$$

In this context, hypothesizing a model implies an explicit definition of F. In that F is a function of two independent variables, F describes a three-dimensional binding surface: the first axis being the concentration of ligand, the second being the concentration of inhibitor, and the third being the concentration of bound ligand. When thought of in this manner, it is clear that a single displacement isotherm describes only a small portion of the binding surface. It was based on this concept that Rothman and coworkers (3) suggested that analysis of a binding surface would provide more information about F than analysis of an isotherm. In this paper we present a computer simulation study which supports this intuitive notion.

METHODS

In this study we simulated the binding of a ligand, L, and an inhibitor I, to two binding sites, as described by the following equation:

$$B = B_1 \times \frac{L}{L + K_{D1}\left(1 + \frac{I}{K_{I1}}\right)} + B_2 \times \frac{L}{L + K_{D2}\left(1 + \frac{I}{K_{I2}}\right)} \qquad (1)$$

In all simulations presented here, the parameters were fixed to the following values: $B_1 = 0.5$, $B_2 = 0.5$, $K_{D1} = 1$ nM, $K_{D2} = 1$ nM, $K_{I1} =$ 1 nM, and $K_{I2} = 100$ nM. Thus the ligand is assumed to interact with identical dissociation constants at two independent binding sites present in equal concentrations. The inhibitor is fixed to be one hundred-fold selective for one of the binding sites.

An 11-point saturation binding isotherm (L = .1, .2, .4, .8, 1.6, 3.2, 6.4, 12.8, 25.6, 51.2, and 102.4 nM) and displacement curves at various ligand concentrations were generated according to equation (1) and a random 5% error introduced into the data. These sets of data were then simultaneously analyzed according to equation (1) with the aid of MLAB, a curve fitting language which utilizes a nonlinear least square curve fitting algorithim (4). The goodness of fit can be judged by the sum of squares which is the sum of the squares of the differences between observed and predicted points. The information content of the data can be judged from the standard deviations of the parameter estimates.

RESULTS

To ascertain the results obtained with a single displacement isotherm, the 11-point saturation binding isotherm was analyzed with a 51-point displacement isotherm. The displacement isotherm was generated at L = 1.0 nM and with I = 10^x M where x ranged from -10 to -5 at intervals of 0.1. The results shown in Table I show that the parameter estimates were almost identical to those that generated the set of data. This is as expected, since only a 5% error was introduced into the data. However, the standard deviations of the parameters were quite large, indicating a large uncertainty as to the exact values of the parameters.

The next simulation considered the effect of two displacement isotherms, each at different ligand concentrations, on the parameter standard deviations. Displacement isotherms were generated at L = 1.0 nM and 10.0 nM with I = 10^x M where x ranged from -10 to -5 at intervals of 0.2. Thus, two 26-point displacement isotherms were considered, leaving the total number of points almost identical to the previous analysis. As shown in Table II, the parameter standard deviations were considerably lower than those resulting from analysis of a single displacement curve. Similar results were obtained with two displacement isotherms with L = 0.1 nM and 10 nM, or L = 0.1 nM and 1.0 nM, and with three, 18-point displacement curves at L = 0.1 nM, 1.0 nM, and 10.0 nM.

DISCUSSION

The results reported here appear perplexing at first glance. Analysis of a single displacement isotherm with 51 points led to the correct parameter estimates but with very large parameter standard deviations. Our interpretation of

TABLE I

Analysis of a Saturation Binding Curve with a Single Displacement Isotherm

PARAMETER	PARAMETER VALUE	PARAMETER STANDARD DEVIATION
B_1	0.499	2.551
B_2	0.501	2.545
K_{D1}	1.006	10.262
K_{D2}	0.994	10.122
K_{I1}	0.951	4.813
K_{I2}	99.486	509.663

An 11-point saturation binding curve was analyzed by MLAB simultaneously with a 51-point displacement curve (at L = 1.0 nM) for the parameters of the two-site model (equation 1).

TABLE II

Analysis of a Saturation Binding Curve with Two Displacement Isotherms

PARAMETER	PARAMETER VALUE	PARAMETER STANDARD DEVIATION
B_1	0.498	0.014
B_2	0.501	0.015
K_{D1}	0.971	0.054
K_{D2}	1.024	0.075
K_{I1}	1.019	0.082
K_{I2}	101.767	8.927

An 11-point saturation binding curve was analyzed with two 26-point displacement isotherms (L = 1 nM and 10 nM) by MLAB for the parameters of the two-site model (equation 1).

these results is that equally good fits can be otained with a variety of parameter values. For example, with B_1 = 0.45 and B_2 = 0.55, an equally good fit could be obtained by adjusting the values of the other parameters. When an additional displacement isotherm from another section of the binding surface is considered, then there is less room for adjusting the parameters, and thus their standard deviations are much lower.

The methodology we describe here involves analysis of multiple displacement isotherms of a single inhibitor. DeLean and coworkers (2) showed that simultaneous analysis of single displacement isotherms of several inhibitors also results in more accurate parameter estimates. These two approaches to experimental design are, in some respects, complementary. The binding surface approach requires that the ligand binding assay provide clean data at widely spaced ligand concentrations. We recently showed that slide-mounted sections of minced rat brain is a suitable preparation for this approach (5). The methodology described by DeLean et al. (2) requires clean data at only a small range of ligand concentration. Thus, depending upon the experimental situation, either method could be used. When possible, we prefer to use the binding surface approach since this provides considerable information about the interaction of a single inhibitor with the receptor without having to consider additional drugs whose mechanism of interaction with the receptor may differ.

We describe in this paper an advanced experimental design which utilizes sophisticated methods of data analysis. As we point out in the INTRODUCTION, the methodology used in a scientific investigation is determined by the question under consideration. The techniques described here and in a previous paper (3) should prove useful for studies of complex receptor mechanisms as well as questions related to multiple receptors.

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NALOXONE AND LONG TERM POTENTIATION OF HIPPOCAMPAL CA3 FIELD POTENTIALS IN VITRO

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ABSTRACT

The effects of naloxone on potentiation of CA3 pyramidal cell field potentials induced by tetanization of the mossy fiber pathway was studied in the in vitro guinea pig hippocampal slice preparation. Naloxone in nanomolar concentrations prevented the development of long term potentiation and it is concluded that an opioid peptide is probably involved in the generation of the potentiation.

INTRODUCTION

Hippocampal mossy fibers arise from granule cells in the dentate gyrus and terminate in characteristic synapses on dendrites of CA3 and CA4 pyramidal cells (5,6,10,12,18). The mossy fiber terminals and the CA3 pyramidal cells are arranged in discrete laminae. Electrical stimulation of the mossy fiber pathway elicits a field potential in the CA3 pyramidal cell layer that is correlated with the synaptic activation of these cells (3,4). Following low frequency tetanizing (LFT) stimulation of the mossy fiber pathway, the CA3 pyramidal cell layer field potential is potentiated for up to several hours in the in vitro preparation (1,15,21). This is referred to as long term potentiation (LTP). The mechanism of CA3 pyramidal cell potentiation is believed to be in part due to disinhibition through a recurrent feedback loop involving inhibitory interneurons (15,21).

Potentiation of pyramidal cell field potentials similar to LTP can be produced pharmacologically in the in vitro preparation by addition of either bicuculline or opiates (9,13). Bicuculline is believed to act as a postsynaptic antagonist of the presumed recurrent inhibitory interneuron transmitter, GABA (9,13). Opiates on the other hand are believed to depress the responsiveness of this interneuron (13). Additionally opiates do not increase the size of mossy fiber evoked excitatory postsynaptic potentials nor do they have a direct effect on CA3 pyramidal cells (13). Like LFT, the ultimate effect of bicuculline and opiates on pyramidal cells may be disinhibition.

The transmitter of the mossy fiber to CA3 pyramidal cell synapse may be an excitatory amino acid (2,11,16,20). However, studies have shown that the mossy

fiber pathway may also contain an opioid peptide (8,14,19). It is conceivable that while normal stimulation of the mossy fiber pathway results in release of an excitatory transmitter at the mossy fiber-CA3 pyramidal cell synapse, LFT may cause the release of an opioid peptide, resulting in the depression of the inhibitory interneuron. The consequence would be LTP of the CA3 pyramidal cell layer field potential. If this case is true then naloxone should have no effect on the normal CA3 pyramidal cell layer field potential, but should block LTP.

METHODS

Experiments were performed on 18 male or female guinea pigs. Animals were anaesthetized with ether, decapitated and 450 μm sections of the hippocampus were prepared as described by Dingledine, Dodd and Kelly (7) using an oscillating tissue slicer. Sections were maintained in a recording chamber (17) at 33° - 35°C and allowed one hour to equilibrate. The artificial cerebral spinal fluid (ACSF) contained NaCl, 124 mM; KCl, 5 mM; NaH_2PO_4, 2.25 mM; $MgSO_4$, 2 mM; $CaCL_2$, 2 mM; $NaHCO_3$, 26 mM; D-glucose, 10 mM. Field potentials were recorded using a glass electrode filled with ACSF (2-10 MΩ). The filter bandwidth was 0.1 Hz to 3 KHz. The recording electrode was placed in the CA3 pyramidal cell layer. Synaptic responses were evoked using two large tipped (~ 50 μ m) glass pipettes placed along the mossy fiber pathway in the CA4 dentate hilus region (Fig. 1A). Current pulses of 40 to 100 μA, 200 μsec at 0.25 Hz were routinely used. These stimulation parameters gave an approximately half maximal response amplitude.

Field potential amplitudes vary between preparations and it becomes increasingly difficult to elicit LTP with increasing time after decapitation. Therefore all field potential values were converted to percent of control response and a time controlled experimental protocol was used. Stimulation and recording began 60 minutes after decapitation. Stable field potentials were recorded for 15 minutes and then 4 to 6 waveform averages of 16 consecutive field potential responses to mossy fiber stimulation were generated over a 10 minute control period. The amplitude of the field potential was computed by measuring the amplitudes of the negative-going population spike and the preceding and following maximum positive amplitudes of the field potential (Fig. 1B).

In control experiments waveform averages were computed every 5 minutes for 60 minutes. The value measured at 60 minutes was compared to the average of the values taken during the 10 minute control period to give change in field potential amplitude over time (NS, for no stimulus, Fig. 1C). To induce LTP three LFT's (15 Hz for 15 sec) were given at 5 minute intervals. In control LTP experiments LFT's were given immediately after the 10 minute control period and waveform averages computed 4 minutes after each LFT and 10, 15, 20, 25 and 30 minutes after the last LFT. In experiments in which naloxone (Endo Laboratories) was added to the ACSF, 4 to 6 waveform averages were computed during the 10 minute control period as before, then the normal ACSF was exchanged for ACSF with naloxone for 15 minutes. If there was no alteration in the amplitude of the field potential then a series of 3 LFT's were given as before with comparable times for recording waveform averages. In all experiments only one slice per animal was used and all data had been collected between 75 and 140 minutes after decapitation.

RESULTS

Figure 1C illustrates the results of these experiments. Three experiments were performed for each procedure. In the absence of any LFT's (NS, for no stimulus, Fig. 1C) the amplitude of the evoked field potential did not change over

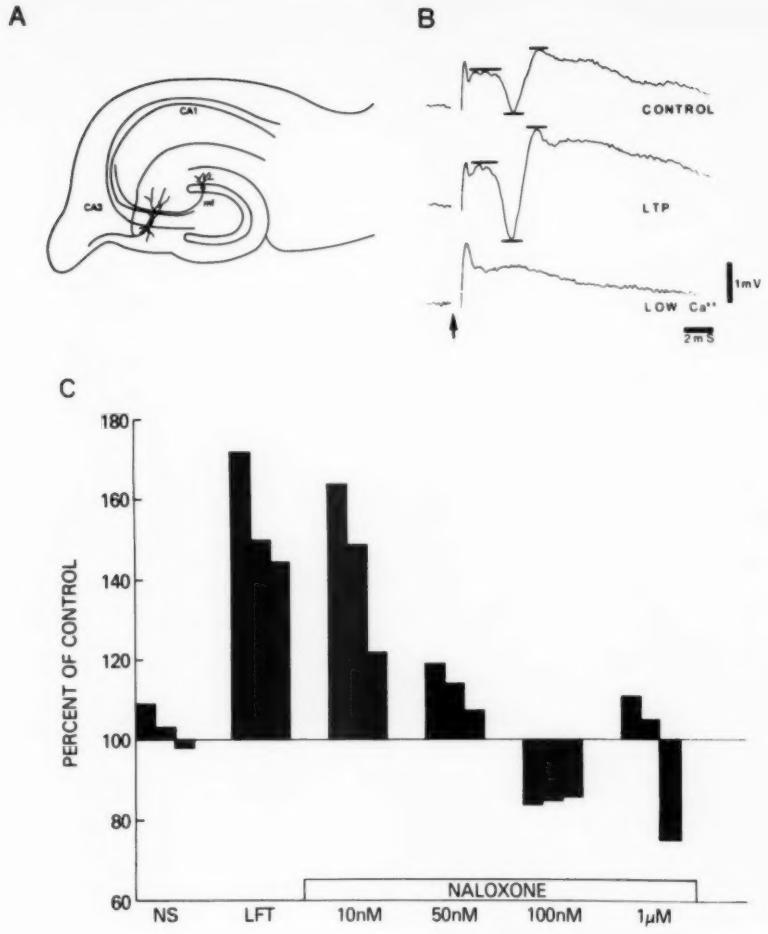

Fig. 1 (A) Diagramatic representation of the hippocampal tissue slice illustrating the mossy fiber pathway (mf) and the CA3 to CA1 pyramidal cell layer. Stimulating electrodes were placed along the mossy fiber pathway and field potentials recorded in the CA3 pyramidal cell layer. (B) Waveform averages of CA3 pyramidal cell field potential to 16 consecutive stimuli during control period, long term potentiation (LTP) and low (0.1 mM) calcium. The horizontal lines indicate the points used to determine the amplitude of the field potential. (C) Bar histogram of CA3 pyramidal cell layer field potential, expressed as percent of control values. Each bar in each cluster of bars represents value obtained for one slice preparation. The following experimental procedures were used: 60 minutes after control period, no LFT (NS, for no stimulus); 20 minutes after third LFT in normal artificial cerebrospinal fluid (LFT); 20 minutes after third LFT in artificial cerebrospinal fluid with 10 nM, 100 nM and 1 μM naloxone.

the time course of the experiment ($\bar{x}$ = 103% ± 5.5 S.D.). A series of 3 LFT's consistently produced LTP (LFT, Fig. 1C, $\bar{x}$ = 155% ± 15 S.D.). Following each LFT there was an initial potentiation of the field potential lasting for about 30 seconds followed by a profound depression lasting up to 2 to 3 minutes. LTP however was evident after each LFT in the waveform averages computed at 4 minutes. Addition of naloxone to the ACSF did not alter the initial potentiation - depression sequence. However, naloxone did cause a dose-dependent depression of LTP. The waveform average following LFT in the presence of 10 nM naloxone increased to an average 145 percent of control (± 21 S.D.). In the presence of 50 nM naloxone the increase was to an average 113 percent of control (± 6 S.D.) and the averaged waveform decreased to an average 85 percent of control (± 1 S.D.) in the presence of 100 nM naloxone. In 1 μM naloxone the waveform was 97 percent of control (± 19 S.D.). In the 3 experiments in which 1 μM naloxone was added, the evoked field potential was monitored for an additional 30 minutes after returning to normal, drug free, ACSF. This procedure did not result in any alteration of the averaged waveform.

DISCUSSION

The evidence clearly suggests that naloxone in nanomolar concentration prevents the development of LTP of CA3 pyramidal cell field potentials following LFT of the mossy fiber pathway. Since naloxone is a specific opioid antagonist it can be concluded that an opioid peptide is probably involved in the generation of this LTP.

Opioid peptides, as discussed above, have no effect on the passive membrane properties of CA3 pyramidal cells, do not influence the size of evoked excitatory postsynaptic potentials and reduce inhibitory postsynaptic potentials (13). Since the site of action of opiates in the CA3 region appears to be the inhibitory interneuron, the origin of CA3 LTP is most likely the result of a depresssion of inhibitory interneuron activity by the evoked release of an opioid peptide.

Physiologically two systems appear to be present in the mossy fiber pathway; a low threshold naloxone-insensitive system between mossy fibers and CA3 pyramidal cells and a high threshold naloxone-sensitive system between mossy fibers and CA3 inhibitory interneurons. There is currently no evidence to suggest whether the two proposed transmitter systems are present in the same mossy fibers or in separate fibers. A detailed electron microscopy study coupled with immunocytochemical techniques may be required to resolve this issue.

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IMMUNOHISTOCHEMICAL LOCALIZATION OF NEUROPHYSIN AND OXYTOCIN IN THE SHEEP CORPORA LUTEA

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ABSTRACT

Antisera raised against neurophysin, oxytocin and vasopressin were used, in conjunction with the immunofluorescence procedure, to localize the neuropeptides in corpora luteal tissue collected from sheep at estrus. Specific immunostaining for neurophysin and oxytocin was observed in all the giant cells of the corpora lutea. In the series of corpora lutea examined staining for vasopressin was not observed.

INTRODUCTION

It is becoming increasingly apparent that the localization of neurophysin and the associated neurohypophysial hormones are not confined to the structures of classical hypothalamo-neurohypophysial system i.e. in nerve terminals of the posterior pituitary gland and within axonal projections from the supraoptic and paraventricular nuclei. Neurophysin-oxytocin- and vasopressin-like immunoreactivities have been demonstrated in the pineal gland of cattle (1) while vasopressin- and oxytocin-containing fibres are found in the pineal gland and subcommissural organ of the rat (2). In a recent publication from this laboratory immunoreactive neurophysin was localized in the corticotrophs of the anterior pituitary gland as well as in the cells of the arcuate nucleus of the rat (3).

The results from physiological studies have suggested that oxytocin may play an important role in luteolysis of sheep, cows and goats (4-8). That the corpus luteum, itself may be responsible for the synthesis of oxytocin has been suggested (9-11). This paper reports on the immunohistochemical localization of oxytocin and neurophysin in the corpus luteum of the sheep ovary.

MATERIALS AND METHODS

Corpora lutea were collected from sheep near the period of luteolysis and fixed by immersion in Bouin's fluid for 14 days. The dehydrated and paraffin embedded tissues were

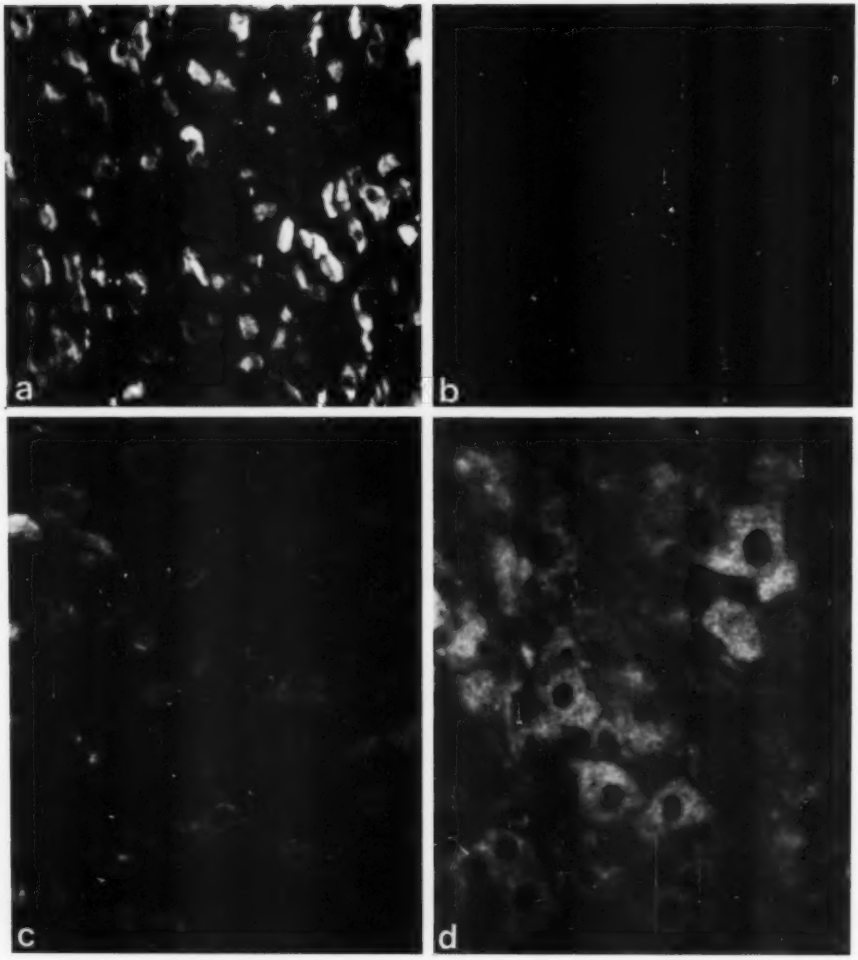

Fig 1a-d.

Immunofluorescence localization of (a,c) neurophysin and (d) oxytocin in cells of the sheep corpus luteum. In (b), the anti-neurophysin serum had been preadsorbed with neurophysin for 24 hr at 4°C prior to use in the immunofluorescence procedure a, b x 226; c, 362; d x 400.

prepared for immunofluorescence histology as previously described (3). The 6μ thick sections were hydrated to phosphate buffered saline (PBS) and treated with either rabbit porcine neurophysin-II serum (diluted 1:80 in PBS), affinity purified rabbit anti-oxytocin serum (diluted 1:40 in PBS) or rabbit anti-vasopressin serum (diluted 1:40 in PBS) (12). The procedure employed for the primary and subsequent histochemical reactions are the same as that reported (3). Controls to establish the specificity of the immunofluorescence were carried out by replacing the antisera with either, PBS or normal rabbit serum. The antisera were also adsorbed with the corresponding antigen (500 μg/ml) for 24 hr at 4°C prior to use in the staining reacton.

RESULTS AND DISCUSSION

All the giant cells of the sheep corpus luteum stained positively for both neurophysin (Fig 1a,c) and oxytocin (Fig 1d). Control reactions in which the antisera was adsorbed with the antigen were negative (compare Fig 1a with Fig 1b). Of the six corpora lutea that were examined, none of the tissues gave a positive stain with the anti-vasopressin serum.

These observations confirm that the cells of the sheep corpus luteum contain material cross-reactive against neurophysin and oxytocin antisera. This supports our findings by radioimmunoassay that extracts of the sheep corpus luteum contain oxytocin and the oxytocin-associated neurophysin (13) (W.B.Watkins, L.G.Moore, V.J.Choy, unpublished observations). In the bovine corpus luteum and human ovary both oxytocin and vasopressin have been partially identified (10,11). That vasopressin-containing cells could not be observed in the sheep corpus luteum may either be a reflection of a species variation or the time during the estrus cycle during which the tissues were collected.

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CATABOLISM OF BOMBESIN IN THE INTERSTITIAL FLUID OF THE RAT STOMACH

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The aim of this study was to develop a method to examine the catabolism of neuropeptides in the tissue fluids that bathe specific regions in the alimentary tract of the conscious animal. Two bundles of dialysis fibers were surgically implanted in the submucosal region of the gastric corpus in the anesthetized rat. After recovery from surgery, one set of fibers was perfused with ^{125}I - Tyr4 bombesin 14. Some molecules of peptide diffused into the tissues and were catabolized by enzymes therein. The catabolites were collected in the second dialysis fiber and fractionated by high pressure liquid chromatography. Bombesin 14 was catabolized to iodo-tyrosine and three unidentified peptides. The catabolites did not bind with a carboxyl-terminal specific antiserum and, as this is the biologically active region of bombesin, were devoid of any biological activity.

INTRODUCTION

Bombesin is a peptide of 14 amino acids that was first isolated from the skin of the european frog (1). A series of peptides sharing the carboxyl-terminal heptapeptide with amphibian bombesin since have been isolated from the porcine fundus (2) and the proximal small intestine of the dog (3). In the alimentary tract and the central nervous system of mammals bombesin-like immunoreactivity has been identified in nerve fibers (4) and the peptide may be considered to be a putative neurotransmitter. As such bombesin may act locally within the tissues without the need to enter the general circulation and act as a hormone. Furthermore, its catabolism may be local and within the interstitial fluid (ISF) that bathes its secretory and target cells. Previously, the release of locally acting substances into the ISF of the stomach has been examined by implanting hollow dialysis fibers in the stomach tissues and collecting the dialysate (5). Now a similar approach has been used to study the inactivation of bombesin in the rat stomach.

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METHODS

Principle

This is shown in figure 1.

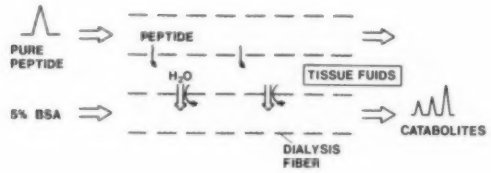


Figure 1. The principle of tissue fluid dialysis.

If a hollow dialysis fiber is placed in ISF and perfused with a radio-labelled peptide some of the peptide molecules will enter the tissue fluids where they may be catabolized enzymically. The catabolites can be collected into a second, parallel dialysis fiber and then separated and quantified by high pressure liquid chromatography (HPLC). The collection of catabolites into the second tube is facilitated if it is filled with a hypertonic, non diffusable solution relative to the ISF because the resulting osmotic flow of water will aid the diffusion of the peptide fragments.

Dialysis fibers

Fibers of the following characteristics were used: source, Spectrum Medical, Los Angeles, California; material, regenerated cellulose; molecular weight exclusion, 6,000 daltons; diameter, 200 μm. For implantation into animals and for *in vitro* studies six fibers, each 6cm long, were glued into silicone tubes with a silicone based glue leaving a 1.5 cm length of exposed fiber (Figure 2).

Figure 2. Dialysis fibers glued to silicone tubes.

Kinetic studies

The feasibility of the approach was established by in vitro experiments. Firstly, a bundle of fibers was placed in a jacket filled with 3 ml of 0.2% bovine serum albumin (BSA) in 0.9% NaCl (to represent ISF) to which 125 I- Tyr 4 bombesin 14 was added (about 50,000 $cpm.ml^{-1}$). The dialysis fibers were filled with a solution of 0, 2.5, 5 or 7.5% BSA in NaCl, which was replaced with a fresh solution every 1,2,3,4,5,6 or 10 min. The dialysate was counted. The optimal conditions for collection of label were when the fibers were filled with 5% BSA for 5 min periods and these conditions were used for all animal studies. In a second study two sets of fibers were placed in a jacket (volume 1.0ml) filled with 0.2% BSA in 0.9% NaCl. One bundle of fibers (the delivery set) was perfused with iodinated Tyr 4 bombesin 14 in water (about 0.5µCi) at a rate of 10µl.min for 40 min. The second set (for collection) was filled with 5% BSA in 0.9% NaCl that was replaced with a fresh solution every 5 min. Samples obtained from the jacket and from the second bundle of fibers were counted.

Animal Studies

Surgical preparation

Male Sprague Dawley rats (250-350 g) were used. Surgery was performed cleanly on rats anesthetized with ether. Two sets of dialysis tubes were implanted in the submucosa of the gastric corpus. An incision was made through the serosal muscle layers to the submucosa, the fibers were placed side by side in the wound and the muscle sutured over the fibers. The silicone tubes were tunnelled subcutaneously to the neck (Figure 3).

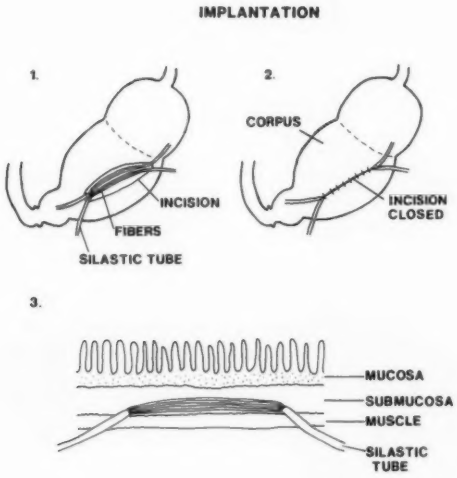


Figure 3. Surgical implantation of dialysis fibers in the gastric submucosa.

Experiments

Experiments commenced on conscious, healthy animals usually 5 days after surgery. They were gently restrained during the 2 to 3 h studies. Iodinated peptide (about 0.5μCi) was perfused through one set of tubes at $10\mu l.min^{-1}$ for 40 min to pulse tissues with the label. The second set of tubes was filled with 5% BSA in 0.9% NaCl to collect any catabolites. The dialyzed fluid was replaced at 5 min intervals, collected on ice into 50μl glass capillary tubes and frozen until chromatography.

Chromatography

Peptides were fractionated by HPLC using a reverse-phase C-18 column (Waters Radial Compression Separation System 5μm, 8 mm 10cm). The column was equilibrated with 0.1% trifluoracetic acid and bound peptides eluted using increasing concentrations of acetonitrile.

Peptides

Tyr 4 bombesin 14 (Pennisula) was iodinated by a modification of the chloramine T procedure as described previously (6). The iodinated peptide was purified by sequential molecular exclusion and cation exchange chromatography. Before use, the homogeneity of each batch of label was assessed by HPLC.

Antibody binding

The ability of each catabolite to bind with a carboxyl terminal antibody (1078) was established by previously described methods (6).

Histology

After a series of experiments animals were killed and the tissues around the fibers were fixed in Bouin's fluid and processed for light microscopy.

RESULTS

Kinetic studies

When dialysis fibers were filled with 5% BSA in 0.9% NaCl, labelled bombesin fully equilibrated within 5 min (figure 4). The equilibration time was longer if they were filled with 0.9% NaCl alone or with 2.5% BSA but was not improved by 7.5% BSA. Perfusion of a set of fibers with bombesin label in water resulted in the rapid accumulation of label in the jacket solution to plateau within 10 min. Label then accumulated in the second set of tubes (figure 5).

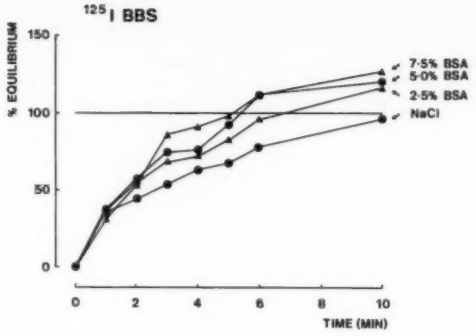


Figure 4. Shows that labelled bombesin rapidly equilibrates across dialysis membrane when fibers are filled with protein solution (one typical experiment).

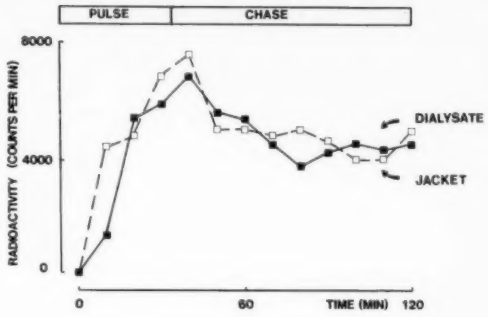


Figure 5. Following perfusion of one set of fibers with bombesin label the peptide rapidly accumulates in the jacket solution and then in the second set of fibers filled with protein solution (one typical experiment).

Animal studies

Of the label perfused through the fibers in the rats, about 85% was recovered and did not enter the animal. Of that entering the stomach tissues usually 40% was recovered in the collection tubes. The amount of label collected increased during the 40 min pulse period to peak after 60 min and then declined over the following 60 min (figure 6). Pools (20 min) of the dialysate were chromatographed.

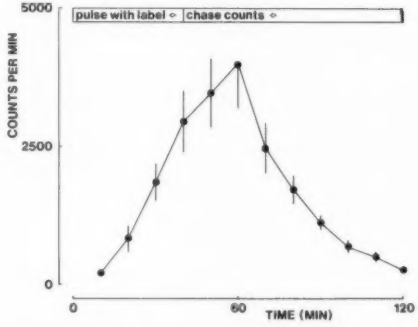


Figure 6. Shows the change in the amount of raioactivity collected in the dialysis fibers with time. Mean ± standard error (n=6).

The HPLC elution profiles of the catabolized bombesin collected in a typical experiment are shown in figure 7. Intact bombesin eluted 46 min after starting the chromatogram and iodotyrosine eluted after 23 min. Peaks A, B and C ran after 25, 29 and 36 min, respectivley. Only intact bombesin bound with the carboxyl-terminal specific antiserum. Changes in the relative sizes of each peak with time are shown in figure 8a,b. Intact peptide was slowly catabolized producing free tyrosine and mainly A. As yet the identities of A, B and C are unknown. Almost 10% of the original peptide, bombesin 1-14, remained intact after 120 min.

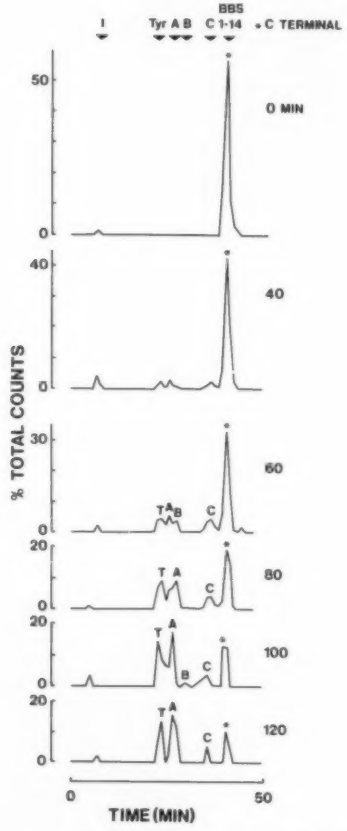
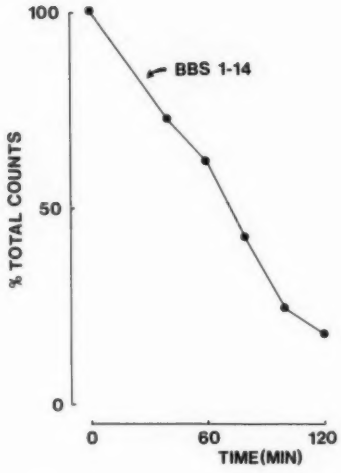
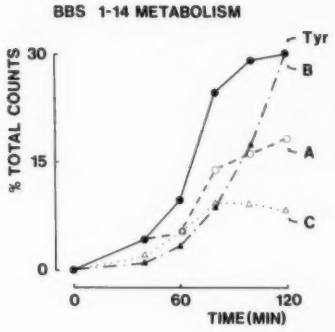


Figure 7. Elution profile of catabolites from C-18 HPLC column in one typical experiment on one rat. Samples of dialysate were collected at 30,60,80,100 and 120 min after starting the experiment. The peptides bound to the column were eluted with a gradient of increasing amounts of acetonitrile run at a 0.5% -min^{-1} from 15 to 35% over 40 min. Fractions of 1 ml were collected.
* indicates ability to bind carboxyl-terminal antibody.



(a)



(b)

Figure 8 a, 8b. Shows how the integrated peak sizes change with time after starting the experiment. Each point is a mean from 6 animals. Standard errors are: bombesin 1-14, 4.6-7.4; Tyr, 2.0-6.6; A, 0.7-3.4; B, 0.5 -3.0; C, 0.2-1.9.

Histology

Implantation of the dialysis fibers was accompanied by an inflammatory response which was characterized by the presence of some mononuclear and polymorphonuclear leucocytes in the immediate vicinity of the fibers (figure 9). The response did not become well developed until at least 1 to 2 weeks after implantation and yet the pattern and proportions of the catabolites produced were the same a day after surgery as 2 weeks later.

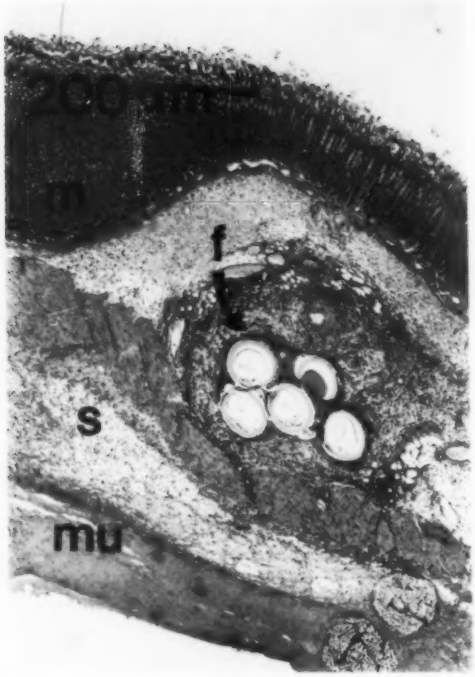


Figure 9. Photomicrograph of fibers 2 weeks after implantation. f= fibers, m=mucosa, s=submucosa, mu=muscle.

DISCUSSION

A new technique has been described to examine the catabolism of a neuropeptide in the interstitial fluid of the stomach. The results show that bombesin 1-14 is catabolized in the submucosa of the gastric corpus of rats to 4 main products: free iodotryosine and 3 as yet unidentified peptides, A, B, and C. Only the intact peptide, bombesin 14, bound to the carboxyl-terminal antibody and since the carboxyl-terminal nonapeptide is the biologically active portion of the bombesin molecule (7,8), all of the other catabolites must have been devoid of biological activity.

The bombesin-related peptides are confined to nerve fibers in the stomach of the rat and are considered putative neurotransmitters or neuromodulators (4). As such they probably act locally on adjacent cells and, by analogy with the classical cholinergic or adrenergic neurotransmitters, may be catabolized and inactivitated locally after secretion. This study demonstrates the local inactivation of a neuropeptide in the conscious animal.

It is not known whether bombesin label was catabolized intracellularly, by membrane bound peptidases or by soluble peptidases in the interstitial fluid. Others have demonstrated potent peptidase activity in the cytoplasm and in subcellular fractions (9,10) but the significance of cytoplasmic catabolism is uncertain for a peptide may not need to enter cells to be inactiviated. Intact synaptasomes catabolize bombesin 14 to produce mainly the 1-7 and 7-14 fragments (11) possibly by a membrane-bound enzyme system. A soluble peptidase activity has been identified in a proteinaceous colloid that accumulates in the pituitary cleft under certain conditions (adrenalectomy or estrogen treatment) (12) and possibly such activity exists in extracellular fluid of the brain and alimentary tract. Whether a specific peptidase enzyme exists for each neuropeptide or whether they function to control the peptide concentrations at the target cell is unknown.

Examination of peptide catabolism using tissue fluid dialysis has several advantages. Firstly, catabolism can be investigated locally in specific regions of the body in conscious animals. Other studies in intact animals have relied upon examining the clearance of a peptide from the general circulation and the appearance of catabolites in blood. Such studies may be of relevance if a peptide acts as a circulating hormone in the classical sense, but are of little relevance to those peptides, including bombesin, that are putative neurotransmitters. Furthermore, it is very difficult to assess the contribution of different tissues or organs to the catabolism of an intravenously administered peptide but the dialysis technique could supply such information. Secondly, the technique can be used to identify the physiologically important steps in peptide breakdown. Investigation of catabolism in vitro cannot possibly account for the subtle regulation of an enzymic activity that is likely to occur in the conscious animal so that the physiological relevance of certain catabolic steps is difficult to define. Also, the study of inactivation by crude homogenates or cellular fractions may give misleading results because the peptide may be degraded by enzymes inside cells in with which it does not normally come into contact. The technique does have a disadvantage because the implantation of dialysis fibers in tissues produces an inflammatory response and contribution of inflammatory cells to catabolism of the peptide cannot be excluded. However the pattern of catabolism observed one day after surgery was similar to that observed several weeks later when the inflammation was more severe. Furthermore, white cells from whole blood slowly break down neuropeptides such as neurotensin (13). It is possible that mammalian bombesin is processed differently than the iodinated amphibian peptide that was used in the present study. The definitive study of the catabolism of mammalian bombesin in tissue fluids must await the availability of the tritrated peptides and the fragments thereof so that the catabolic products can be identified. It is anticipated that the technique of interstitial fluid dialysis may contribute to these studies.

ACKNOWLEDGEMENTS

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NEUROHYPOPHYSIAL PEPTIDES AND THE HIPPOCAMPUS. I. VASOPRESSIN IMMUNOREACTIVITY IN THE RAT HIPPOCAMPUS

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ABSTRACT

Immunocytochemical studies have demonstrated that nerve fibres containing immunoreactive vasopressin project to many areas of the central nervous system. In the present investigation, the presence of immunoreactive arginine vasopressin (IR-AVP) in the hippocampus of Wistar rats was confirmed by radioimmunoassay. The vasopressin content of the dorsal hippocampus was 30.3 ± 7.3 pg IR-AVP/mg soluble protein (mean ± SEM, n=9) and that of the ventral hippocampus was 81.4 ± 8.3 pg IR-AVP/mg soluble protein (n=9), while tissue from the cerebral cortex contained no detectable vasopressin. That the immunoreactivity was due to vasopressin was confirmed by its absence in hippocampal or cortical tissue from homozygous Brattleboro rats, which are genetically unable to synthesize vasopressin.

INTRODUCTION

The hypothalamic neurones which synthesize the neurohypophysial hormones oxytocin and vasopressin project to many areas of the mammalian central nervous system (2,11). In the rat the projections to rostral regions of the brain consist primarily of fibres containing arginine vasopressin (AVP) immunoreactivity, while fibres to caudal regions and to the spinal cord usually contain immunoreactive oxytocin instead of vasopressin (1). The innervation of the hippocampus is controversial. According to Buijs (1,2), nerve fibres containing immunoreactive vasopressin (IR-AVP) reach the hippocampus by two pathways. One projection passes via the fornix into the dorsal hippocampus and continues into the ventral hippocampus (2), while the other passes through the amygdala and enters the ventral hippocampus directly (1). Sofroniew and Weindl (11) have confirmed that fibres containing IR-AVP pass through the amygdala into the ventral hippocampus, but they could not detect any fibres in the

dorsal hippocampus. Moreover, while Buijs (1) reported that the immunoreactive fibres terminated among the dendrites of the pyramidal cells, the fibres described by Sofroniew and Weindl (11) appeared to terminate in the molecular layer of the dentate gyrus and in the hilar region.

In the present study the vasopressin content of the ventral and dorsal portions of the hippocampus was measured by radioimmunoassay.

METHODS

Sample Preparation

Samples of brain tissue were obtained from 9 male Wistar rats (220-430 g) and 7 female Brattleboro rats (182-250 g) homozygous for diabetes insipidus ("HoDI rats"). The HoDI rats were determined to be homozygous by their inability to concentrate urine during dehydration. Each rat was anaesthetized (urethane 1.0-1.5 g/kg body weight, i.p.) and decapitated, and its brain was immediately removed and placed in cold saline. The hippocampi were dissected free and were divided into approximately equal dorsal and ventral portions, and a sample of similar size was taken from the dorsal surface of the cerebral cortex. The samples were kept on ice throughout the following procedures, including homogenization, extraction, and radioimmunoassay. Right and left portions of each tissue were combined, homogenized in 0.2 M acetic acid (15) with a Teflon coated pestle in a Potter-Ehlvehjem assembly and centrifuged at 15,000 g for 30 minutes. The supernatant was lyophilized and stored at -20°C. In addition to the individual samples, tissues from 6 Wistar rats (410-480 g) were combined to form a "pooled hippocampal" sample and a "pooled cortical" sample, and cortical tissue from 3 HoDI rats (185-215 g) was combined as "pooled HoDI cortex".

Acetone/petroleum ether extraction

Vasopressin was extracted from the lyophilized samples by a modification of the method of Robertson et. al. (10). Each sample was dissolved in 2.0 ml of 2.0 M acetic acid, and 200 µl was removed for protein determination (Biorad Protein Assay Kit I, with bovine gamma globulin as standard). Acetone (2.0 ml) was added to the remaining 1.8 ml to precipitate the proteins, and the sample was mixed on a Vortex mixer and centrifuged (1200 g for 50 minutes). The supernatant was decanted and saved, and the pellet was resuspended in 1.0 ml acetone and centrifuged again, the two supernatants were combined, and the pellet was discarded. Lipids were then removed by the addition of 5.0 ml of petroleum ether. The two phases were mixed on a Vortex and allowed to separate for at least one hour before the ether (upper phase) was aspirated and discarded. The thin film at the interface was allowed to remain with the lower phase.

After the addition of 100 µl of 0.2 M acetic acid, the sample was evaporated to less than its original volume under a stream of nitrogen gas to remove the acetone and any remaining petroleum ether. The sample remained in the ice bath during the evaporation. The final solution was lyophilized and stored at -20°C until assayed.

Radioimmunoassay

The radioimmunoassay was a modification of one described previously (9). The antiserum ("R-20") was obtained from an HoDI rat immunized with lysine vasopressin (LVP) conjugated to bovine serum albumin by the carbodiimide technique (6). The antiserum is specific for vasopressin, AVP being about twice as effective as LVP, and does not cross react significantly (<1%) with oxytocin, vasotocin, isotocin, or angiotensin I. The vasopressin used as a standard in the assay was the posterior pituitary extract kindly provided by Dr. R.E. Weitzman of Harbor General Hospital, Torrance, California. The extract was standardized against synthetic AVP (Spectrum). Iodinated AVP was prepared as described by Weitzman and Fisher (14), based on the Chloramine-T technique (7).

All assays were performed in triplicate. The reagents were dissolved in 0.15 M phosphate buffer (pH 7.2), and the non-specific binding was measured in tubes containing buffer in place of antiserum. Bound and unbound fractions were separated by the addition of dextran-coated charcoal. The sensitivity of the assay, calculated as the amount of hormone required to produce a 20% depression in binding, was 0.24 ± 0.02 pg (mean ± SEM), the intra-assay coefficient of variation at 1.0 pg was 3.3% (n=4), and the interassay coefficient of variation at 2.5 pg was 9.8% (n=5).

RESULTS

The amount of IR-AVP detected in the rat brain tissues is shown in Table I. Vasopressin was detected in all nine of the ventral hippocampal samples and in all but one of the nine dorsal hippocampal samples from the Wistar rats. The amount of vasopressin in the ventral hippocampus (81.4 ± 8.3 pg IR-AVP/mg soluble protein, mean ± SEM) was 2.7 times greater than in the dorsal hippocampus (30.3 ± 7.5 pg IR-AVP/mg soluble protein). In contrast to this, none of the cortical samples from the Wistar rats contained detectable vasopressin (<6.2 pg IR-AVP/mg soluble protein). No immunoreactivity was detected in any of the tissue samples from the HoDI rats, which are genetically unable to synthesize AVP (13), confirming that the immunoreactive material in the Wistar rats was in fact AVP.

TISSUE	n	IR-AVP (pg/mg soluble protein)
HoDI V. HIPP.	6	n.d.
HoDI D. HIPP.	5	n.d.
HoDI CORTEX	2	n.d.
WISTAR V. HIPP.	9	81.4 ± 8.3
WISTAR D. HIPP.	9	30.3 ± 7.5
WISTAR CORTEX	7	n.d.

TABLE I. VASOPRESSIN CONTENT OF RAT BRAIN TISSUES

All values have been corrected for extraction loss as described in the text. The values are means ± SEM. AVP was not detected (n.d.) in the Wistar cortex or in the HoDI tissues. The hippocampus was divided into ventral (V. HIPP.) and dorsal (D. HIPP.) portions. Each sample represents the tissue from one rat. Two Wistar cortex samples and four HoDI cortex samples were used to compute the correction factor, and one HoDI D. HIPP. sample was destroyed.

Synthetic AVP (11.25 pg) was added to each of 4 HoDI cortical samples and two Wistar cortical samples. Recovery of IR-AVP in these samples was 45.7 ± 4.2 (mean ± SEM, n=6), and the values in Table I have therefore been corrected appropriately. Since the AVP was added to the tissue immediately after dissection, the correction factor should compensate for the loss of hormone during all stages of tissue processing.

Normally the dissecting dish and instruments were rinsed after each dissection. One brain from an HoDI rat, however, was deliberately dissected in the saline remaining from the dissection of a Wistar rat brain to determine whether vasopressin leaking from the severed pituitary stalk could be contaminating the tissues during dissection. The tissue samples from this rat did not contain detectable vasopressin. This finding, and the absence of vasopressin in the Wistar cortical samples, indicate that contamination could not have accounted for the vasopressin detected in the Wistar hippocampal tissues.

To allow larger amounts of material to be assayed, brain tissue from several rats was combined. Pooled hippocampal tissue prepared from six male Wistar rats contained 236 pg IR-AVP/mg soluble protein (value corrected as described above), 2.9 times as much as the single ventral hippocampal samples, and

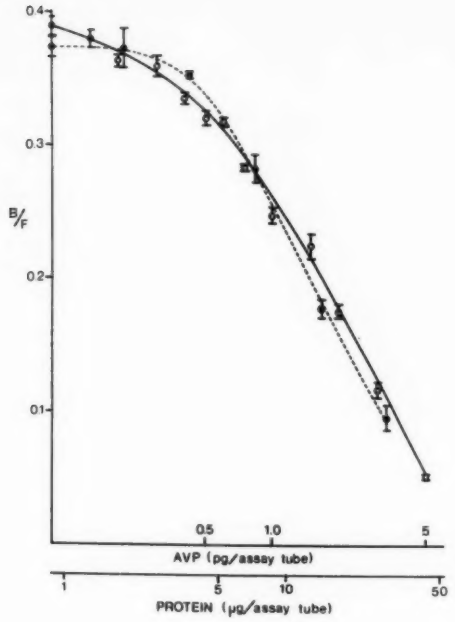

Fig. 1 Serial dilution of hippocampal tissue. The curves produced by serial dilution of the AVP standard (open circles) and of the pooled Wistar hippocampal tissue (closed circles) are shown. The ordinate shows the ratio of bound to free radioactivity, and the abscissae represent the amount of AVP standard or of tissue (expressed in µg soluble protein/assay tube). The relation between the two abscissae is arbitrary and was chosen to facilitate comparison. Values are plotted as means ± SEM, n=3).

pooled cortical tissue from the same rats contained 26.3 pg IR-AVP/mg soluble protein, even though IR-AVP was not detectable in the single cortical samples. Pooled cortical tissue from three HoDI rats failed to yield detectable IR-AVP. The curve produced by serial dilution of the pooled tissue was parallel to that produced by serial dilution of the AVP standard (Fig. 1), as would be expected if the immunoreactive material in the tissue was immunologically identical to AVP.

DISCUSSION

Radioimmunoassay of rat brain tissue confirmed that IR-AVP is present in the hippocampus and indicated that the ventral portion of the hippocampus contains more of this peptide than does the dorsal portion. This finding is consistent with the reports by Buijs (1,2), which suggest that the immunoreactive fibres project primarily but not exclusively to the ventral hippocampus, but it is not in agreement with the work of Sofroniew and Weindl (11), who found the dorsal hippocampus to be devoid of fibres containing vasopressin. The discrepancy between the findings of Buijs and those of Sofroniew and Weindl may be due to differences in the specificity of their antisera. Sternberger (12) has suggested that the immunoreactivity demonstrated by immunocytochemistry may not be due to the AVP nonapeptide itself but to larger fragments of the AVP precursor. The fibres described by Buijs (1,2) as passing through the fornix to the pyramidal cell layer were found primarily but not exclusively in the ventral hippocampus and apparently contain a fragment of the AVP precursor which does not react with the antiserum used by Sofroniew and Weindl (11). The fibres passing through the amygdala to the dentate gyrus however, are found only in the ventral hippocampus (11) and contain material that can be detected by either antiserum.

It is not clear why the pooled hippocampal tissue contained more IR-AVP than the individual samples. The rats from which the pooled tissue was obtained were older than those used for the individual samples, and analysis of the data from the individual samples suggested that tissues from older rats contain more IR-AVP than do tissues from young rats, but the difference was not sufficient to explain the quantity of IR-AVP in the pooled sample. The IR-AVP found in the pooled cortical sample is also surprising, although both Dogterom et. al. (3) and Hawthorn et. al. (8) reported small amounts of IR-AVP in rat cortical tissue. Immunoreactive fibres have been reported in the entorrhinal cortex (2), but the tissue in the present study was taken from the dorsal surface of the cortex, which appears to be devoid of such fibres.

Previous reports of immunoassayable AVP in the rat hippocampus have not been entirely in agreement either with each other or with the present study. George and Jacobowitz (4) were unable to detect any IR-AVP in the hippocampus, or in any other extrahypothalamic tissue, but their assay was not sensitive enough to measure the levels reported here. Using more sensitive assays, Hawthorn et. al. (8) found 27 pg IR-AVP/mg total protein in the whole hippocampus and Dogterom et. al. (3) reported 30 pg IR-AVP/mg dry weight and 58 pg IR-AVP/mg dry weight respectively in the "posterior" and "anterior" portions of the dorsal hippocampus. Exact comparisons with the present study are impossible because of differences in the way the

results are expressed, but the amount of IR-AVP detected by Hawthorn et. al. (8) appears to be about four fold greater than in the present study, and the amount reported by Dogterom et. al. (3) is at least 10 times as great. Both in the present study and in the report by Dogterom (3) the immunoreactive material was not present in HoDI rats and must therefore be derived from the AVP precursor, but it is not certain that the material consisted exclusively of AVP itself. It is likely that mammalian tissues contain more than one AVP-like molecule - two immunoreactive variants of AVP containing 11 amino acids have recently been reported (5) - and antisera with different specificities for these molecules will therefore measure different amounts of immunoreactivity. Further studies will be required to characterize completely the immunoreactive molecule(s) in the hippocampus.

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NEUROHYPOPHYSIAL PEPTIDES AND THE HIPPOCAMPUS. II. EXCITATION OF RAT HIPPOCAMPAL NEURONES BY OXYTOCIN AND VASOPRESSIN APPLIED IN VITRO

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ABSTRACT

Neurohypophysial peptides were applied by superfusion to rat hippocampal slices. The peptides, arginine vasopressin, lysine vasopressin, arginine vasotocin and oxytocin, increased the activity of 88% of spontaneously active cells in the CA1 region and induced firing in many neurones that were not spontaneously active. The peptide sensitive cells appeared to be pyramidal cells rather than interneurones. The four peptides were found to be of roughly equivalent potency, producing a reversible, dose-dependent response in the range 10^{-9} to 10^{-6}M. Most of the cells were tested with more than one peptide and were always found to respond either to all or to none of them. The analogue [7-glycine]oxytocin and the deamino, dicarba derivatives of oxytocin and vasopressin were about as active as the parent compounds, but the oxytocin fragment prolyl-leucyl-glycinamide had no effect, and desglycinamide vasopressin was extremely weak. Responses to the peptides could be blocked by "specific" antagonists. The results suggest that all of the peptides are acting upon a single class of receptor.

INTRODUCTION

The neurohypophysial (NHP) peptides oxytocin and vasopressin are reported to be involved in many aspects of brain function, including catecholamine (16) and serotonin (2) turnover, the retention of conditioned behaviour (5) and the response to fever (9). In some experiments oxcytocin has the same effect as vasopressin (28); in others it has the opposite effect (5) or may be relatively inactive (31). Several studies (2,14,16) have implicated the hippocampus in the effects of the

NHP peptides. The evidence that nerve fibres containing immunoreactive vasopressin project to the hippocampus was discussed in the preceding article (29). Hippocampal fibres containing immunoreactive oxytocin have been reported by Buijs and Swaab (6), but they are not as numerous as those containing vasopressin, at least in the ventral hippocampus, and their existence could not be confirmed by Sofroniew and Weindl (27).

Unpublished experiments from this laboratory have shown that hippocampal neurones could be excited by vasopressin applied iontophoretically in vivo, but the results were erratic, and a detailed study of analogues and antagonists using this method was impossible. In the work described in the present paper, we have studied the effects of the NHP peptides on individual neurones in slices of rat hippocampus by adding the peptides to the bathing medium.

METHODS

Hippocampal slices were prepared from 41 male Wistar rats (160-360 g) and from two female Brattleboro rats (180-190 g). The Brattleboro rats were determined to be homozygous for diabetes insipidus by their inability to concentrate urine during dehydration. Each rat was decapitated, its brain was removed, and one hippocampus was quickly dissected free and cut into 400 μm transverse slices, approximately in the plane of the hippocampal lamella (26), on a McIlwain tissue chopper. The slices were immediately transferred to a nylon net in an acrylic recording chamber. The lower surface of each slice was in contact with a thin film of oxygenated (95% O_2/5% CO_2) medium that flowed along the bottom of the chamber at a constant rate (about 0.5 ml/minute), and the upper surface was exposed to the humidified O_2/CO_2 mixture. The chamber was maintained at a constant temperature of 33°C. The oxygenated medium, and media with added peptides, were stored in plastic containers and allowed to flow by gravity into the recording chamber. The basic medium contained (mM): NaCl 124, KCl 5, $NaHCO_3$ 26, $NaHPO_4$ 1.25, $MgSO_4$ 2, glucose 10, and $CaCl_2$ 2. In some experiments the peptides were dissolved directly in the medium. In others the lyophilized peptides were first dissolved in 20-100 μl of acidified saline (1.0 mM HCl in 140 mM NaCl), and the acid solution was then diluted 500-1000 fold with medium and neutralized (1.0 mM NaOH in 140 mM NaCl). All control and peptide solutions were acidified and neutralized in the same way in any given experiment. The peptides included arginine vasopressin ("AVP", Sigma, grade VIII), lysine vasopressin ("LVP", Sigma, grade IV), arginine vasotocin ("AVT", Spectrum), oxytocin ("OXT", Sigma), [1-deamino-1,6-dicarba]arginine vasopressin ("ddAVP", Beckman), [1-deamino-1,6-dicarba]oxytocin ("ddOT", Beckman), prolyl-leucyl-glycinamide ("PLG", Beckman), and somatostatin ("SST", Serono). Dr. M. Manning of the Medical College of

Ohio, Toledo, Ohio, kindly provided AVP, [desglycinamide-8-arginine]vasopressin ("DGAVP"), [7-glycine]oxytocin ("Gly^7OT"), [1-(ß-mercapto-ß,ß-cyclopentamethylenepropionic acid),2-(O-methyl)tyrosine]arginine-vasopressin ("$d(CH_2)_5$ Tyr(Me)AVP"), and [1-(ß-mercapto-ß,ß-cyclopentamethylenepropionic acid),2-(O-methyl)-D-tyrosine,4-valine]arginine-vasopressin ("$d(CH_2)_5$-D-Tyr(Me)VAVP").

The response to the peptides was monitored by extracellular unit recording, using 4M NaCl filled glass micropipettes (0.5-4.0 M Ω) positioned in the stratum pyramidale of the CA1 region. A platinum wire running the length of the recording chamber served as the reference electrode. A bipolar stimulating electrode (62 µ twisted Nichrome wire), positioned in the Schaffer collateral/commissural pathway, was used to study evoked responses.

RESULTS

The four NHP peptides - AVP, LVP, AVT and oxytocin - had a pronounced excitatory effect when applied onto rat hippocampal slices (Fig. 1). The effect was dose dependent when the peptides were applied in concentrations of 10^{-9} to 10^{-6} M (Fig. 2). Not all cells responded to the peptides (see below), and some that appeared to respond were rejected from further study for such technical reasons as small spike amplitude or excessive background noise. The data below are based on a study of 57 cells that responded unambiguously to repeated applications of peptide.

The effects of the four peptides were qualitatively and quantitatively similar. Most neurones were tested with more than one of these peptides and in all cases responded either to all of the peptides tested or to none of them. No cell was found which responded to one NHP peptide but failed to respond to another. By contrast somatostatin (SST) appeared to affect a different population of cells. One cell responded to both SST and oxytocin, two cells sensitive to the NHP peptides failed to respond to SST, and one cell insensitive to AVP was excited by SST (Fig. 3A).

Several synthetic analogues of the NHP peptides were also tested. PLG, an oxytocin fragment known to potentiate the behavioural effects of L-Dopa (15), was found to be totally inactive in tests on four cells sensitive to oxytocin and to AVT (Fig. 3B). The analogue Gly^7OT, which is reported to be active at oxytocin receptors but not at vasopressin receptors (20), was tested on four peptide sensitive cells and was found to be a potent agonist (Fig. 4), but DGAVP, tested on two peptide sensitive cells, produced only a very weak excitation of one cell (Fig. 4A) and failed to excite the other. The oxytocin analogue ddOT was found to be about as effective an

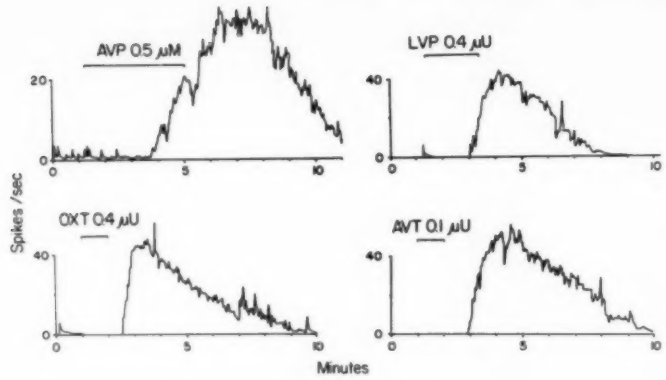

Fig. 1 Effect of NHP peptides on hippocampal neurones in vitro. The firing rate is indicated on the ordinate in spikes per second and time on the abscissa in minutes. Medium was allowed to flow past the slice continuously; during the period indicated by the horizontal bars, medium containing the peptide was substituted for the control medium. The concentration of the peptide is indicated above the bar.

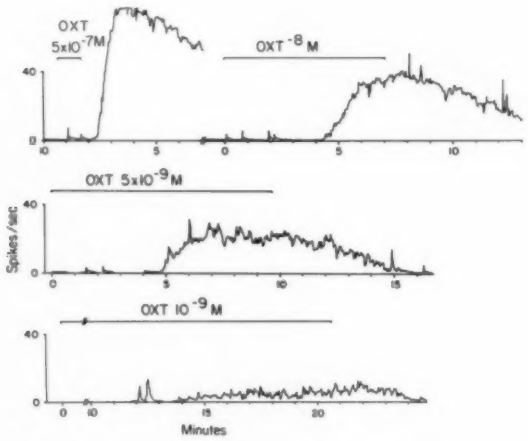

Fig. 2 Response of a hippocampal neurone to low concentrations of oxytocin. The four responses were recorded from the same cell. The response was both stronger and more rapid at higher concentrations.

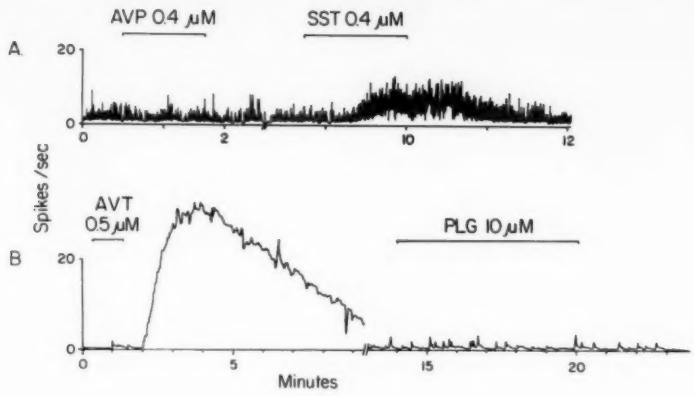

Fig. 3 Effect of SST and PLG on hippocampal neurones. A. The effect of SST on a neurone insensitive to AVP. B. Failure of an AVT- and oxytocin-sensitive neurone to respond to PLG.

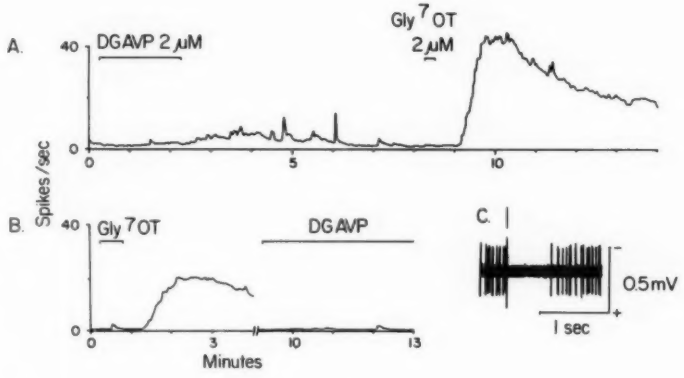

Fig. 4 Effect of Gly^7OT and DGAVP on hippocampal neurones. A. Tracing from a cell which responded to oxytocin and to Gly^7OT but which responded only weakly to DGAVP. B. Tracing from a neurone which responded to Gly^7OT but not to DGAVP. C. Stimulation of the Schaffer collateral/commissural pathway resulted in an inhibition of the Gly^7OT induced activity of the cell in trace B. Two sweeps are superimposed.

agonist as oxytocin. This analogue is protected against enzymatic attack at the N-terminus, where it lacks an amino group, and at the disulfide bond, which is replaced by a dicarba ($-CH_2-CH_2-$) linkage. An aminopeptidase activity said to be specific for oxytocin has been found in rat brain tissue (7), but its ability to activate or inactivate oxytocin in the hippocampal slice would appear to be minimal. The equivalent AVP analogue, ddAVP, was also active (1 cell), but its activity was not compared to that of AVP.

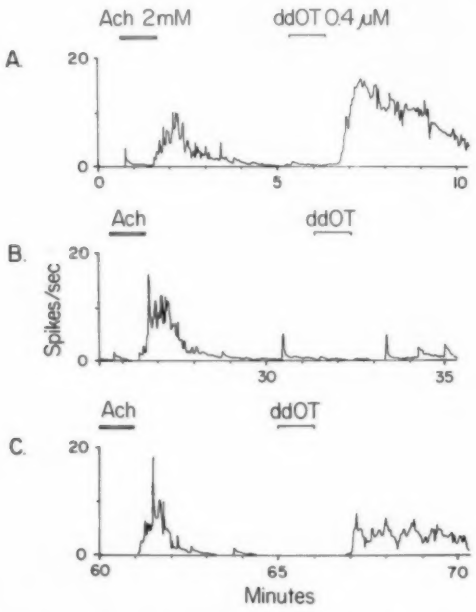

Fig. 5 Response to ddOT blocked by $d(CH_2)_5$ Tyr(Me)AVP. The three tracings are from the same cell, before (A), during (B), and after (C) exposure to the antagonist at a concentration of 0.1 µM. The antagonist was applied for 10 min before the start of B and had been discontinued 20 min prior to the start of C. The response to ddOT, but not that to acetylcholine (ACh), was almost completely blocked by the antagonist. Partial recovery from the effects of the antagonist is apparent in C.

The response to all of the NHP peptides could be blocked by prior application of the antagonist $d(CH_2)_5$Tyr(Me)AVP. The specificity of the antagonist is apparent in Fig. 5: prior application of the antagonist at a concentration of 0.1 µM almost completely blocked the response to 0.4 µM ddOT but had no effect on the response to 2 mM acetylcholine. The

antagonist was also tested against AVP, LVP, AVT and oxytocin and was effective against each of these peptides. A second antagonist, $d(CH_2)_5$-D-Tyr(Me)VAVP, was tested against AVP, oxytocin, ddOT and Gly^7OT (Fig. 6). Like $d(CH_2)_5$Tyr(Me) AVP, this antagonist consistently blocked the effect of the peptides but not that of acetylcholine. The two antagonists were tested on a total of 15 cells and always blocked the responses to all of the peptides.

Repeated brief applications of peptide did not cause peptide-sensitive cells to become less rsponsive, provided that the firing rate of the cell was allowed to return to control levels between applications. When subjected to longer applications of peptide however, the cells lost their sensitivities rather quickly. As can be seen in Fig. 7, a cell whose response to oxytocin declined during superfusion thereafter was insensitive to AVP as well, a finding that suggests that both peptides act on the same receptor.

Few spontaneously active cells were encountered in the slice preparations, but 7 of 8 spontaneously active cells tested (88 %) were peptide sensitive. Most of the peptide-sensitive cells included in this study had little or no spontaneous activity but could be induced to fire by an application of peptide - the cells were discovered by exposing the slices to one of the peptides and then searching for active cells. The peptide-sensitive cells were located in stratum pyramidale of area CA1, and stimulation of the Schaffer collateral/ commissural pathway resulted in a 200-600 msec inhibition of their peptide-induced activity (Fig. 4C) indicating that they were pyramidal cells rather than interneurones (18). No peptide-sensitive cells were found in areas CA3 or CA4 or in the dentate gyrus, but these regions were not explored extensively. There was no obvious difference in responsiveness between slices from the ventral hippocampus, where most hippocampal fibres containing immunoreactive vasopressin are found (27), and those from the dorsal hippocampus. As reported previously by Mühlethaler et. al. (22), the peptide sensitivity of the cells does not depend on prior exposure of the rat to AVP, since sices from homozygous Brattleboro rats, which are genetically unable to synthesize vasopressin (30), were as responsive as those from Wistar rats.

DISCUSSION

The present study demonstrates that oxytocin and vasopressin can cause reversible, dose-dependent excitations of neurones in the CA1 region of the rat hippocampus and that the excitations can be prevented by substances that block NHP peptide receptors. Excitation of rat hippocampal neurones by vasopressin applied *in vitro* has been reported previously by Mühlethaler et al. (22), and the present study confirms their

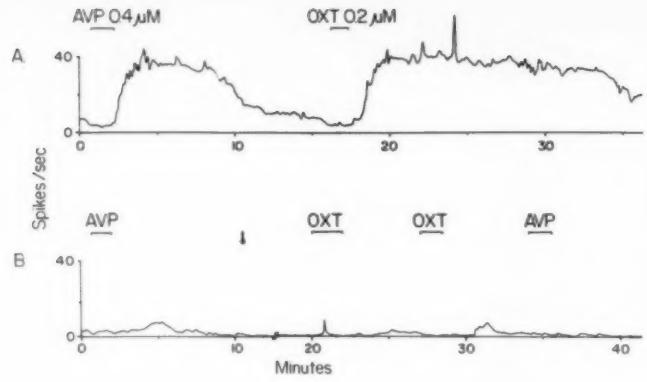

Fig. 6 Response to AVP and to OXT blocked by $d(CH_2)_5$-D-Tyr(Me)VAVP. The response of a cell to 0.4 μM AVP and to 0.2 μM OXT before (A) and during (B) application of $d(CH_2)_5$-D-Tyr(Me)VAVP is shown. The antagonist was applied at a concentration of 0.04 μM for 5 min before the start of trace B and was discontinued at the time indicated by the arrow.

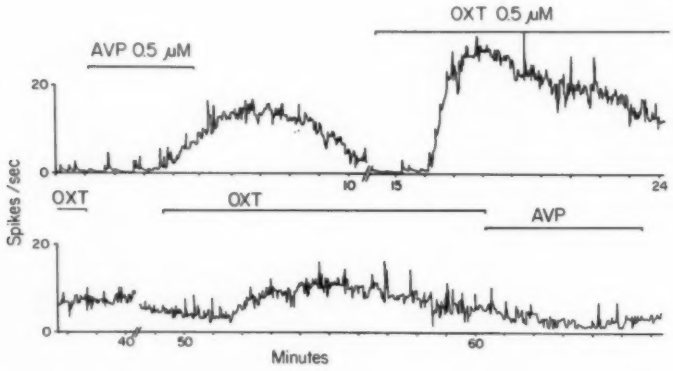

Fig. 7 Decline in responsiveness of a cell subjected to continuous exposure to oxytocin. There is a 14 min gap between the end of the upper and the start of the lower tracing. The effect of a 25 min application of oxytocin is shown; the response to oxytocin began to decline within 5-6 min. The slice was then washed with control medium for 10 min (min 39-49; note discontinuity in the time scale), but the cell thereafter remained relatively unresponsive to both oxytocin and AVP. This cell had previously responded consistently to repeated brief applications of oxytocin and AVP.

findings. The two reports are in agreement that AVP, LVP and oxytocin excite CA1 cells, that DGAVP is weak or inactive, that $d(CH_2)_5$Tyr(Me)AVP blocks the effect of vasopressin and that slices from Brattleboro rats respond as readily as do those from normal rats. The earlier study (22) was confined to the ventral hippocampus, but the work reported here suggests that neurones in the dorsal hippocampus may be equally sensitive to the peptides.

The fact that the NHP peptides were found to excite cells under experimental conditions does not, however, prove that the physiological function of the peptides is excitatory. Inhibitions would not have been detected in the present experiment because of the low level of spontaneous activity, but Dreifuss and Mühlethaler found in a preliminary study (13) that vasopressin depressed the firing rate of some hippocampal neurones and excited others when the activity of the cells was monitored by intracellular electrodes. This is in contrast to their extracellular studies (22) described above, in which vasopressin produced only excitations despite high background rates of spontaneous activity. A similar situation exists in regard to SST, which was found to excite hippocampal neurones in the present experiments and in a study by Dodd and Kelly (12) but which inhibited them in the experiments reported by Pittman and Siggins (23). Whatever the reason for these discrepancies, it is probably best to interpret the peptide-induced changes in firing rate as indicating only that the tissue possesses peptide receptors, not as establishing any physiological function for the peptides.

The nature of the receptors on the CA1 neurones is not entirely clear. Mammalian NHP peptide receptors are traditionally divided into four types according to their bioactivity. Vasopressin acts primarily on the vasopressor (VP or V_1) receptor and on the antidiuretic (AD or V_2) receptor, while oxytocin affects the uterotonic receptors and those of the mammary gland (4). A fifth type of receptor, the HF-R or PA vasopressin receptor, has recently been described (8,10); it is involved in the release of haematological factors and is thought to be located in the central nervous system (10). The antagonist $d(CH_2)_5$Tyr(Me)AVP (17) blocks the VP but not the AD vasopressin receptors and also prevents the uterotonic response to oxytocin. Unlike $d(CH_2)_5$Tyr(Me)AVP, $d(CH_2)_5$-D-Tyr(Me)VAVP is an effective antagonist at both the VP and the AD receptors (21), but its effect on oxytocin receptors has not been reported. In accord with these criteria, the hippocampal response to AVP may be mediated by the VP receptor, but it could not be due to the AD receptor since it was blocked by $d(CH_2)_5$Tyr(Me)AVP. An action at the HF-R receptor is also unlikely, since Mühlethaler et al. (22) report that CA1 neurones responded only weakly to [1-deamino-8-D-arginine]vasopressin (dDAVP), an agonist active at both the AD

and the HF-R receptors (8,25). The effects of oxytocin and its analogues ddOT and Gly^7OT did not result from an action on vasopressin receptors - Gly^7OT is inactive at VP and AD receptors (20) and oxytocin is inactive at HF-R receptors (8) - but may have involved oxytocin receptors such as the uterine or mammary gland receptors. A simpler explanation however may be that oxytocin and vasopressin act upon the same population of receptors. This interpretation is supported by the fact that the hippocampal neurones were never found to distinguish between the two peptides; cells responding to either of them were always found to be about equally responsive to the other. The demonstration that continuous treatment with oxytocin desensitized a cell to both oxytocin and vasopressin also suggests a common receptor for the two. If this hypothesis is correct, the rat hippocampus must contain an NHP peptide receptor different from the five types defined above. There have been several reports indicating that mammalian tissues contain additional types of peptide receptor - including at least three subtypes of vasopressor receptor (1), an AVT specific receptor (19), a receptor sensitive to desglycinamide vasopressin (24), and a PLG sensitive receptor (15) - but none of these have yet been described in sufficient detail. The broad specificity of the putative hippocampal receptor is similar to that of the NHP peptide receptor in the nervous system of the snail (3), which also responds to oxytocin, AVP, LVP and AVT but not to PLG or to desglycinamide vasopressin.

The hippocampus forms much of the lower surface of the lateral ventricles of the rat, and the excitation of hippocampal CA1 cells by vasopressin might therefore seem to suggest a mechanism for the reported ability of intracerebroventricular injections of vasopressin to increase retention of conditioned behaviour (5). Such a suggestion, however, is not supported by the peptide specificity observed in the present experiment, since oxytocin, which was as effective as vasopressin in stimulating CA1 cells, usually has an effect opposite to that of vasopressin on conditioned behaviour (5). Furthermore, DGAVP, which mimics AVP in promoting retention of conditioned behaviour (11), had little effect on CA1 cells <u>in vitro</u>.

As discussed in the preceding article (29), there appear to be two projections by which fibres containing immunoreactive AVP reach the hippocampus, one to the pyramidal cells and one to the dentate gyrus, and these paths may contain slightly different AVP-like molecules. The two paths may also differ in their effects, since the excitation of hippocampal neurones by AVP was found in the present study to be confined to the pyramidal cells, while the ability of AVP to release serotonin from hippocampal slices is reported to be confined to the dentate gyrus (2).

ACKNOWLEDGEMENTS

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ENKEPHALIN-LIKE IMMUNOREACTIVITY IN *DROSOPHILA MELANOGASTER*

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ABSTRACT

Enkephalin-immunoreactive neurons have been identified in the central nervous system of the fruit fly *Drosophila melanogaster* by immunocytochemical techniques. Analyses of fly extracts by high performance liquid chromatography and radioimmunoassay show a relatively complex pattern of immunoreactive compounds. The most prominent among them has chromatographic properties similar to those of met-enkephalin from which can, however, be distinguished by high-resolution chromatographic techniques.

INTRODUCTION

Enkephalins appear to act as neurotransmitters or neuromodulators in the nervous system of numerous animal species (1-3). While their presence in vertebrates is well documented (3-5),data available for invertebrates are scarce and for the most part obtained by immunocytochemical techniques (6,7). It is generally accepted that the relative simplicity of the invertebrate nervous system makes it a good model for studying peptide neurobiology at the cellular level. Thus, it would seem of interest to ascertain whether opiate peptides, identical or related to those found in higher species, occur also in invertebrates, and, at a later stage, to study the regulation of the expression of genes coding for these neuropeptides. For this reason, we have investigated the presence of these compounds in the fruit fly *Drosophila melanogaster*, an organism easily amenable to genetic analysis.

Immunocytochemistry has the obvious advantage of showing the compounds of interest *in situ* providing a direct morphological evidence of their presence. However, this technique, even in

its more sophisticated form, does not allow an unequivocal identification of the molecular species being visualized. Therefore, it is a definite advantage to combine immunocytochemistry with specific analytical techniques, which may shed more light on the chemical identity of the immunoreactive compounds. This cautionary approach may be specially relevant when dealing with a relatively complex family of closely related peptides such as enkephalins (8-11). We have chosen to combine high performance liquid chromatography (HPLC) with radioimmunoassay (RIA) in order to be able to detect the cross-reaction of the antibodies (raised against vertebrate enkephalin) with any peptide(s) structurally akin to enkephalins which might be present in the tissue extracts (12). The present report attempts a correlation between the enkephalin-like immunoreactivity observed in some neurons of the central nervous system of *D. melanogaster*, and the patterns of immunoreactive compounds detected by HPLC.

METHODS

Drosophila stocks

Fly cultures were kept at 25°C, under standard conditions. For histological studies a *cn bw* homozygous strain, resulting in white color eyes, was used in order to eliminate the eye pigment-induced fluorescence. For HPLC determinations normal or *cn bw* flies were used with identical results.

Immunocytochemistry

Flies were fixed overnight, at 4°C, in 4% paraformaldehyde in phosphate-buffered saline, pH 7 (PBS), washed in 30% sucrose-PBS for 24 hr and sectioned in a cryostat at 10 μm. Sections were incubated with met-or leu-enkephalin antibodies (Immuno-Nuclear Corp.), diluted 1/1000 in a mixture of PBS-0.3% Triton X-100-1 mg/ml bovine serum albumin, at 4°C, for 18 hr, and then washed twice with PBS. Anti-enkephalin reactivity was localized by further incubation in goat FITC-IgG anti-rabbit IgG (Cappel) diluted 1/30 in the above buffer, for 30 min, with two final washes in PBS. Sections were finally mounted in glycerol-PBS (9:1,v/v).

Preparation of fly extracts

Adult flies (head plus thorax) were pulverized in liquid nitrogen in a mortar and homogenized in 10 vol of a solution containing 1 M acetic acid, 20 mM HCl, 0.1% mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride. After centrifugation at 100,000 g, for 30 min, the supernatant was further deproteinized with 10% trichloroacetic acid (TCA). Following a second centrifugation the supernatant was extracted with ether to remove TCA (13). The samples were then lyophilized, dissolved in 0.4 M pyridine-0.5 M formic acid, pH 4, and further processed as described below.

Competition curves

The antigenic similarity between fly peptides and met-enkephalin was tested by comparing the curves obtained by RIA of se-

rial dilutions of both the fly extracts and the synthetic met-enkephalin standard. For these experiments the crude extracts, obtained as described in the previous paragraph, were partially purified by reverse phase cartridges (Sep-Pak disposable cartridges, Waters Assoc. Inc.). The procedure was essentially that of Gay and Lahti (14). Briefly, the cartridges were preactivated with 2 ml of methanol, followed by 5 ml of deionized water and 5 ml of a mixture of 0.5 M formic acid and 0.5 M pyridine, adjusted to pH 3. The sample, in 0.5 ml of this buffer, was injected onto the cartridge. A step gradient of n-propanol (0%, 5%, 10%, 30%) in the formate-pyridine buffer was used to elute the sample at a rate of 1 or 2 drops per second.

To determine that the immunoreactive compound(s) were indeed peptides the extracts were digested with 1 mg/ml pronase (Calbiochem) in 300 µl of 0.05 M Tris-HCl buffer, pH 8.5, at 37°C, for 3 hr. The samples were then boiled for 15 min to inactivate the pronase, centrifuged and processed as above.

HPLC fractionation of fly extracts

HPLC fractionation was carried out in a Waters Assoc. instrument. The column (Microbondapak C 18) was previously calibrated with ^{3}H-enkephalins and ^{125}I-enkephalins as internal markers (tracer enkephalins). Two sets of chromatographic conditions were used :

a) isocratic elution, at 1 ml/min, with 0.4 M pyridine-0.5 M formic acid buffer, pH 4, containing 15% n-propanol. One ml fractions were collected, starting from 2 min after injection time.

b) A very shallow 0-12% n-propanol gradient, running for 2 hr at a flow rate of 0.5 ml/min.

We were careful to use "virgin" columns to avoid the possibility of any memory effect in subsequent chromatographic runs. The recovery of ^{3}H-enkephalin for the entire procedure was 82 ± 4% (n=7) in both cases.

The fractions were lyophylized and analyzed by RIA with either anti-leu-enkephalin or anti-met-enkephalin antibodies, using the corresponding ^{125}I-enkephalin standard (Immuno-Nuclear Corp.). The sensitivity of the assay was 13 fmol per tube. The cross-reactivity of the antisera, as checked in our laboratory, was as follows : leu-enkephalin antiserum, 24% with met-enkephalin; met-enkephalin antiserum, 2% with leu-enkephalin. Neither one exhibited significant cross-reactivity with β-endorphin.

RESULTS

Immunochemical identification of enkephalin-like peptides in the nervous tissue of *Drosophila melanogaster*.

The *in situ* visualization of enkephalin-like immunoreactivity was carried out in frozen sections using a fluorescein-conjugated second antibody. The following results summarize our observations in the adult fly nervous system.

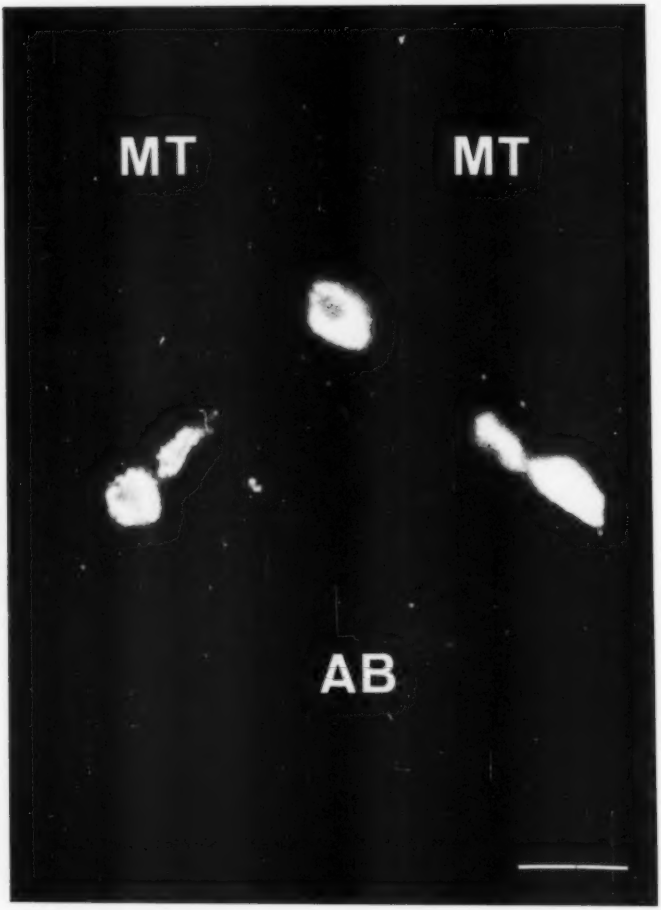

Fig. 1. Horizontal section through the thoracic nervous system of the fruit fly showing five out of the six cell bodies with met-enkephalin antibody immunoreactivity. Mt: Metathoracic; Ab: Abdominal ganglia. Bar = 20 µm.

a) Anti-met-enkephalin antibody: The major part of the fly central nervous system (CNS) shows no reactivity with antibodies against either enkephalin. However, the met-enkephalin antibody labels three different groups of cells within the suboesophageal ganglion in the head. Two of them are located anteriorly and composed of 6-8 cells each, their staining being rather weak. The third group is actually a single cell, very intensely stained, located posteriorly within the ganglion. All these groups are bilateral.

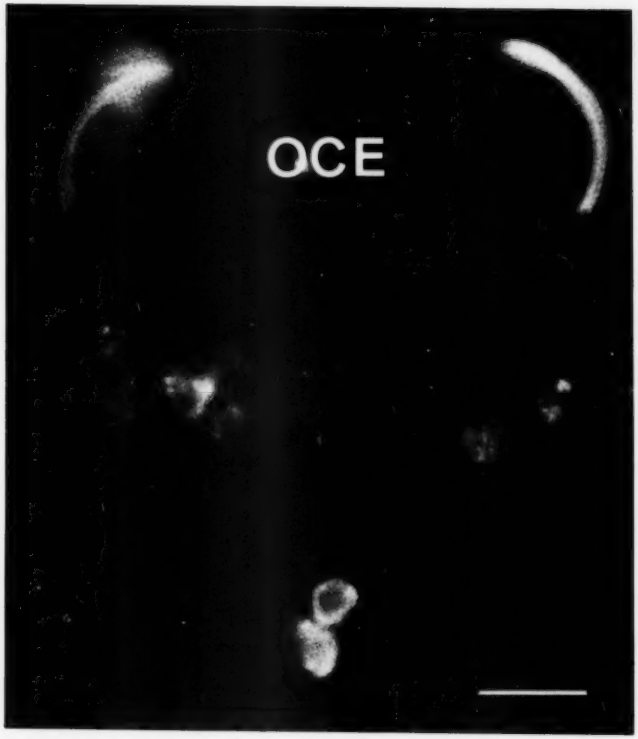

Fig. 2. Frontal section of the adult *Drosophila* brain showing the leu-enkephalin-like immunoreactive cells (bottom of the figure) of the medial groove under the ocelli (OCE). Note the autofluorescence of the cornea in the ocelli and the nonspecific fluorescence of the fat. Bar = 20 µm.

Along the thoracic ganglia, staining can be seen in 1 or 2 cells per segment in the ventral side. In addition, a prominent group of three cells is found at the junction of the metathoracic and abdominal ganglia, on the dorsal side (Fig. 1). All these cells also showed fluorescence in their axons. Finally, some tracts with varicosities were seen running along the stomodeal nervous system, penetrating the brain from the rear at the level of the aortic funnel.

In the larvae of the third instar, groups of immunoreactive cells against met-enkephalin antibody were found in homologous locations : brain and ventral ganglia.

As a control, tissue sections were incubated with met-enkephalin antibody previously adsorbed with an excess of met-enke-

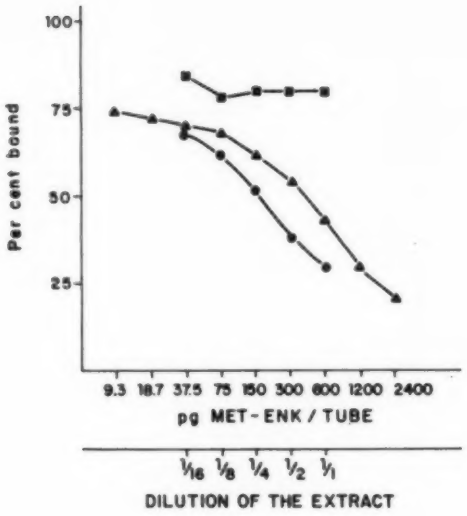

Fig. 3. Competition curves of ^{125}I-enkephalin against fly extracts and the effect of pronase. ● : Serial dilutions of fly extracts; ▲ : Serial dilutions of synthetic met-enkephalin; ■: Fly extract plus pronase.

phalin. Under these conditions no immunoreactivity could be detected in these cell bodies and tracts. By contrast, preincubation of the antibody with leu-enkephalin did not alter the pattern of immunoreactivity. An additional control was carried out by pre-incubating the tissue sections with either fraction number 4 or 10, from the HPLC chromatogram (see below, Fig. 4) and then incubating the sections with met-enkephalin antibody. Either treatment abolished the staining in all tissues.

b) Anti-leu-enkephalin antibody: The anti-leu-enkephalin used showed a higher background than that of the anti-met-enkephalin, the observations being, therefore, less conclusive. Nevertheless, cell bodies with strong labelling were seen in the CNS. These were located in the medial groove of the brain under the ocelli (Fig. 2), and were not detected with the met-enkephalin antibody. In addition, other cell bodies located in similar positions to those seen with the met-enkephalin antibody were also stained with leu-enkephalin antibody, albeit very faintly.

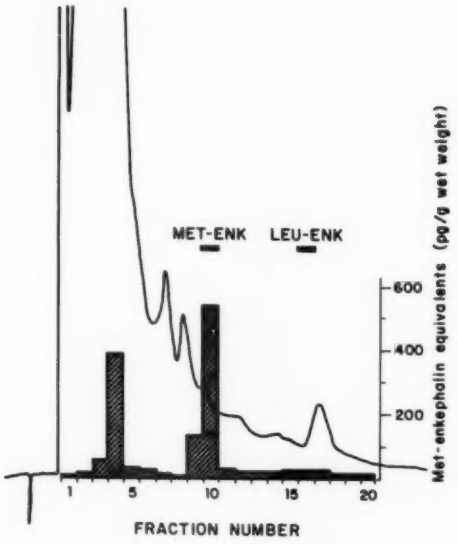

Fig. 4. HPLC fractionation and RIA analysis of fly extracts against met-enkephalin antibody. Bars indicate the fractions where synthetic met- and leu-enkephalins elute. Chromatographic conditions type a (see Methods).

The enkephalin-immunoreactive cells were located in areas where acridine orange and Gomori stains have been found to reveal large cells with cytoplasmic inclusions (15,16), thus suggesting a possible neurosecretory role.

In addition to neural tissues, met- and leu-enkephalin-like immunoreactivity was seen in the gonads, the ejaculatory bulb and accesory gland of the male, and in the peripheral fat.

Biochemical identification of enkephalin-like peptides in *Drosophila melanogaster*:

Competition curves. Tissue extracts of *Drosophila* (head plus thorax) were purified, prior to the RIA, using Sep-Pak disposable

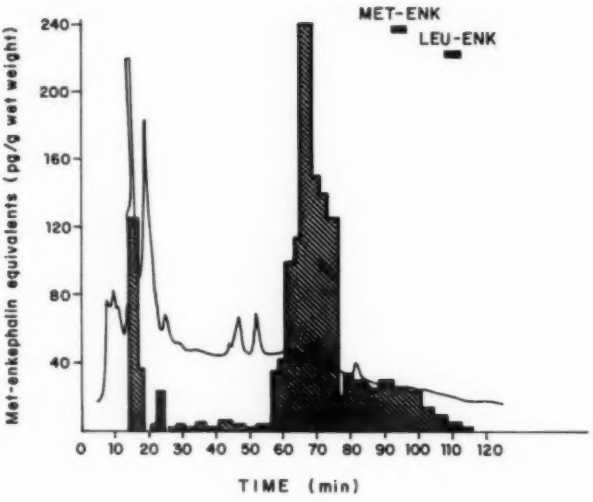

Fig. 5. HPLC fractionation and RIA analysis of fly extracts against met-enkephalin antibody. Bars indicate the fraction where synthetic met- and leu-enkephalins elute. Chromatographic conditions type b. (see Methods).

cartridges. The 10% propanol-fraction containing the enkephalins (as previously checked with ^{3}H-enkephalins) was lyophilized and assayed with met-enkephalin antiserum. The competition curves obtained with serial dilutions of tissue extracts were essentially parallel to those obtained with dilutions of synthetic enkephalins (Fig. 3) pointing to a definite antigenic similarity between the fly peptides and the enkephalin standard. The displacement of bound ^{125}-I enkephalin produced by the extracts is abolished by previous treatment with proteolytic enzymes such as pronase.

HPLC fractionation. Fig. 4 shows the profile of met-enkephalin-like immunoreactivity in fly extracts, fractionated isocratically by HPLC. Over 65% of the total immunoreactivity (expressed in met-enkephalin equivalents and not corrected for recovery losses) corresponds to the fraction where synthetic met-enkephalin elutes. This result would suggest the presence in *Drosophila* of an immunoreactive compound(s) indistinguishable from synthetic met-enkephalin.

The rest of the immunoreactivity (23%) was essentially found in fraction 4, co-eluting with the bulk of the U.V.-absorbing material in the extract. However, the competition curve made with serial dilutions of these early eluting fractions did not parallel the standard curve, suggesting that these compounds are not antigenically similar to met-enkephalin (data not shown).

Under chromatographic conditions providing a higher resolution (see Methods, chromatographic condition b) it was found that the main peak of immunoreactivity was in fact displaced with respect to the tracer met-enkephalin (Fig. 5). This displacement was the same whether the whole extract, or just fraction 10 in Fig. 4, were subjected to this high resolution chromatographic procedure.

Using the anti-leu-enkephalin antibody the overall background of immunoreactivity appears to be much higher in all the fractions. However, a maximum was also observed in fractions 60-70 of the high resolution chromatogram. Although differences in antiserum specificity (see Methods) might account for this fact, we can not rule out at present the possibility that this reflects the presence of a even wider variety of leu-enkephalin-like substances in *Drosophila*.

DISCUSSION

This report describes the detection of enkephalin-like immunoreactive peptides by use of HPLC/RIA and immunohistochemistry in the fruit fly *Drosophila melanogaster*. The *in situ* visualization technique shows definite groups of neurons in the CNS reacting with specific anti-enkephalin antibodies. This does not imply that the immunoreactive cells that we have identified are the only ones containing the peptides that actually exist in the fly's nervous system. For example, Jan and Jan (17) reported the immunohistochemical localization of leu-enkephalin-reactive tracts in the optic glomeruli which we have not found. In fact, if we consider the data from the HPLC and RIA analysis, it is not even clear which of the different immunoreactive compounds are being visualized in these cells. Nevertheless, since the chromatographic analysis shows the presence of peptides with properties very similar to those of vertebrate enkephalins, and the localized cells belong, in all probability, to the neurosecretory class, a moderate confidence seems justified.

From the biochemical point of view, the radioimmunoassay of serial dilutions of partially purified extracts resulted in a binding curve which was essentially parallel to the standard curve for met-enkephalin (Fig. 3) indicating antigenic similarities between this pentapeptide and the immunoreactive material present in *Drosophila*. These antigenic similarities seem to correspond only to the compounds eluting in fraction 10 of Fig. 4, since competition curves made with serial dilutions of the early-eluting immunoreactive material do not parallel the standard

curve. This would be consistent with an interaction of the antiserum with the large amounts of unrelated peptides eluting in the first fractions. On the other hand, the complete loss of immunoreactivity following pronase treatment is a clear indication of the peptidic nature of the compounds under study.

In the isocratic HPLC analysis (Fig. 4) the met-enkephalin immunoreactivity elutes in the same position as synthetic met-enkephalin. However, by increasing the resolving power of the chromatographic procedure we have established that the main component responsible for this immunoreactivity is not identical to met-enkephalin. In fact, only trace amounts of immunoreactivity are finally found in the position of true met-enkephalin. Thus, if met- or leu-enkephalin are present in the *Drosophila* extracts, they occur in very small amounts and are not amenable to quantification with the techniques employed in this study.

We can, therefore, conclude that the main peptide compounds reacting with the antisera against vertebrate enkephalins in *Drosophila* are antigenically related to, but chemically different from the pentapeptides met- and leu-enkephalin.

Further studies are in progress in our laboratories to ascertain whether the *Drosophila* opioid peptides include molecules like met-enkephalin-lys^6 and met-enkephalin-arg^6, or oxidized forms of both penta- and hexapeptides (which would cross-react with anti-met- and anti-leu-enkephalin antibodies and elute ahead of the regular pentapeptide standards (13,18)),or represent new peptide sequences, potentially characteristic of invertebrates.

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*Harvard Univ, Sch Publ Hlth, Toxicol Lab, Boston, Ma 02115, USA
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Pituitary immunoreactive calcitonin-like material: lack of evidence for cross-reactivity with pro-opiomelanocortin

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Univ Glasgow, Stobhill Gen Hosp, Dept Mat Med, Glasgow G21 3UW, Scotland
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*Mt Sinai Hosp & Med Ctr, Dept Med, Div Endocrinol, New York, NY 10029, USA
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Judd, A.M., Hedge, G.A.*
*Virginia Univ, Med Ctr, Dept Physiol, Morgantown, WV 26506, USA
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St Georges Hosp, Sch Med, Dept Physiol, London SW17 ORE, England
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Nippon Roche Res Ctr, Dept Pha Pharmacol, Kamakura 247, Japan
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*Mem Sloan Kettering Canc Ctr, George C Cotzias Lab Neurooncol, 1275 York Ave, New York, NY 10021, USA
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CNR, Ist Psicobiol & Psicofarmacol, Via Reno 1, I-00198 Roma, Italy
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Univ Kentucky, Albert B Chandler Med Ctr, Dept Anat, Lexington, Ky 40536, USA
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*Prince Henrys Hosp, Med Res Ctr, Melbourne, Victoria 3004, Australia
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*Free Univ Amsterdam, Fac Med, Dept Pharmacol, 1081 BT Amsterdam, Netherlands

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Vasopressin is not involved in the catecholamine-induced release of ACTH, alpha-MSH and beta-endorphin from the rat pituitary gland

Randle, J.C.R., Moor, B.C., Kraicer, J.*
*Queens Univ, Dept Physiol, Kingston, Ontario, Canada K7L 3N6
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*Queens Univ, Dept Physiol, Kingston, Ontatrio, Canada K7L 3N6
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Tapia-Arancibia, L., Astier, H.
Univ Montpellier 2, Dept Physiol, Neuroendocrinol Lab, CNRS, ERA 85, F-34060 Montpellier, France
NEUROENDOCRINOLOGY 1983, 37 (2) 166
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CNRS, Physiol Nerveuse Lab, F-91190 Gif Sur Yvette, France
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Univ Birmingham, Sch Med, Dept Physiol, Birmingham B15 2TJ, W Midlands, England
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Douglas Hosp Ctr, Montreal, Quebec, Canada H4H 1R3
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*Vet Adm Med Ctr, Lexington, Ky 40511, USA
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*Polish Acad Sci, Inst Pharmacol, Smetna 12, PL-31343 Cracow, Poland
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Catholic Univ Nijmegen, Dept Zool, 6525 ED Nijmegen, Netherlands
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Univ Fed Rio Grande Sul, Inst Biociencias, Dept Bioquim, BR-90000 Porto Alegre, RS, Brazil
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Maudsley Hosp & Dept Chem Endocrinol, St Bartholomew's Hosp, London, England
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Temple Univ, Dept Psychol, Philadelphia, Pa 19122, USA
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USAF, Radiobiol Res Inst, Dept Behav Sci, Bethesda, Md 20814, USA
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USAF Acad, Dept Behav Sci & Leadership, Co 80840, USA
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Max Planck Gesell, Forsch Grp Reprod Med Klin, Steinfurter Str 107, D-4400 Munster, W Germany
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Nagy A., Graf L., Lajtha A.
Ctr Neurochem, Wards Isl, New York, NY 10035, USA
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Met-enkephalin binding to opiate receptors is not functionally coupled to biodegradation

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Univ N Carolina, Div Clin Pharmacol, Chapel Hill, NC 27514, USA
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Univ Graz, Dept Exptl & Clin Pharmacol, A-8010 Graz, Austria
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Jen M.F., Garzon J., Loh H.H., Lee N.M.*
*Univ Calif San Francisco, Dept Pharmacol, San Francisco, Ca 94143, USA
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Dynorphin-(1-13): The effect of in vivo treatment on opiate bindings in vitro

Mashal R.D., Owen F.*, Deakin J.F.W., Poulter M., Slater R.
*Clin Res Ctr, Div Psychiat, Watford Rd, Harrow HA1 3UJ, England
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*Eli Lilly & Co, Lilly Res Lab, Dept Cent Nervous Syst Res, MC907, 88-4, 307 E McCarty St, Indianapolis, In 46285, USA
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Uniformed Serv Univ Hlth Sci, Neurobiol Res Unit, Bethesda, Md 20814, USA
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Central autonomic effects of dermorphin in conscious rats

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*Harvard Univ, Sch Publ Hlth, Toxicol Lab, Boston, Ma 02115, USA
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Inhibition of asparagine-linked glycosylation of pro-opiomelanocortin in mouse pituitary cells by Di-threo-beta-fluoroasparagine

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Acad Med Sci USSR, Inst Pharmacol, Nervous Syst Pharmacol Lab, Moscow 109801, USSR
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*Hop Eichat, Dept Diabet, F-75877 Paris 18, France
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Janicki, P.K., Habermann, E.*
*Univ Giessen, Audolf Buchheim Inst Pharmakol, D-6300 Giessen, W Germany
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Tetanus and botulinum toxins inhibit, and black widow spider venom stimulates the release of methionine-enkephalin-like material in vivo

Rust, M.
Tech Univ Munich, Klinikum Rechts Isar, Inst Anaesthesiol, D-8000 Munich 80, W Germany
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Niesink, R.J.M., Van Ree, J.M.*
*Rudolf Magnus Inst Pharmacol, State Univ Utrecht, Med Fac, 3521 GD Utrecht, Netherlands
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Ecole Natl Vet Toulouse, Physiol Lab, F-31076 Toulouse, France
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Univ Erlangen Nurnberg, Inst Physiol Chem, D-8520 Erlangen, W Germany
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*Mayo Clin & Mayo Fdn, Dept Pharmacol, Rochester, Mn 55901, USA
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Univ British Columbia, Dept Physiol, Vamncouver, BC, Canada V6T 1W5
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Enkephalingeric control of somatostatin secretion from the perfused rat stomach

Bernatzky G., Doi T., Jurna I.*
*Univ Saarlandes, Inst Pharmakol & Toxikol, D-6650 Homburg, W Germany
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Univ Otago, Dept Pharmacol, Dunedin, New Zealand
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Univ Med & Dent New Jersey, New Jersey Sch Osteopath Med, Dept Med, Div Res, Camden, NJ 08103, USA
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Rockland Res Inst, Ctr Neurochem, Wards Isl, NY 10035, USA
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Univ Sassari, Ist Patol Med 1A, I-07100 Sassari, Italy
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Vet Adm Med Ctr, Med Serv, E Orange, NJ 07019, USA
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Leucine-enkephalin modification of pituitary response to LH-releasing hormone

Iovino M., Rouhani S., Poenaru S.
UER Biomed, Dept Physiol Neuroendocrinol Lab, 45 rue St Peres, F-750270 Paris 06, France
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Fujita Gakuen Univ, Sch Med, Dept Neuropsychiat, Toyoake, Aichi 47011, Japan
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Fujita Gakuen Univ, Sch Med, Dept Neuropsychiat, Toyoake, Aichi 47011, Japan
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Changes of immunoreactive beta-endorphin and somatostatin contents in rat brain after amygdaloid kindling (Abstract)

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Nicholas Copernicus Med Acad, Inst Physiol Grzegorzecka 16, PL-31531 Cracow, Poland
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Effect of enkephalin and naloxone on gastric acid and serum gastrin and pancreatic polypeptide concentrations in humans

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Univ Milan, Sch Med, Dept Pharmacol, Neonatal Serv, I-20129 Milan, Italy
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Plasma beta-endophrin, beta-lipotropin and met-enkephalin concentrations during pregnancy in normal and drug-addicted women and their newborn

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*Brigham & Womens Hosp, Endocrine Hypertens Unit, Boston, Ma 02115, USA
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Beta-endorphin suppresses adrenocorticotropin and cortisol levels in normal human subjects

Petrucha R.A., Goebelsmann U.*, Hung T.T., Haase H.R., Lobo R.A.
*Univ So California, Los Angeles Cty Med Ctr, Womens Hosp, Dept Obstet & Gynecol, Los Angeles, Ca 90033, USA
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Amniotic fluid beta-endorphin and beta-lipotropin concentrations during the 2nd-trimester and 3rd-trimester

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Univ Sherbrooke, Fac Med, Dept Physiol & Pharmacol, Sherbrooke, Quebec, Canada J1H 5N4
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Peripheral vasodilation and plasma extravasation are part of the mechanism of neurotensin-induced hypotension in the anesthetized rat

Theodorsson-Norheim, E., Thor, K., Rosell, S.
Karolinska Inst, Dept Pharmacol, S-10401 Stockholm 60, Sweden
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Fletcher D.R., Shulkes A., Bladin P.H.D., Booth D., Hardy K.J.
Univ Melbourne, Austin Hosp, Dept Surg, Heidelberg, Victoria 3084, Australia
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Cholinergic inhibition of meal stimulated plasma neurotensin like immunoreactivity in man

Wallace, J.L., Chin, B.C., Track, N.S.
Univ Toronto, Dept Surg, Toronto, Ontario, Canada M5S 1A1
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Hypothalamic immunoreactive neurotensin: changes induced by stress

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*Univ Nice, CNRS, Ctr Biochim, F-06034 Nice, France
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Degradation of neurotensin by rat brain synaptic membranes: involvement of a thermolysin-like metalloendopeptidase (enkephalinase), angiotensin-converting enzymes, and other unidentified peptidases

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*Univ Heidelberg, Inst Anat, D-6900 Heidelberg, W Germany
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Neurotensin and substance P immunoreactive nerve endings in the guinea pig carotid sinus and their ultrastructural counterparts

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Univ Lund, Dept Ophthalmol, S-22101 Lund, Sweden
ACTA PHYSIOL SCAND 1983, 118 (3) 215
Substance P fibres in the anterior segment of the rabbit eye

Fandriks, L., Delbro, D.
Gothenburg Univ, Dept Physiol, S-41124 Gothenburg, Sweden
ACTA PHYSIOL SCAND 1983, 118 (3) 301
Neural stimulation of gastric bicarbonate secretion in the cat. An involvement of vagal axon-reflexes and substance P?

Moss, M.S., Basbaum, A.I.
Univ Calif San Francisco, Dept Obstet Gynecol & Reprod Sci, San Francisco, Ca 94143, USA
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The peptidergic organization of the cat periaqueductal gray. II. The distribution of immunoreactive substance P and vasoactive intestinal polypeptide

Stoppini, L., Baertschi, A.J.*, Mathison, R., Barja, F.
*Univ Geneva, Dept Anim Biol, CH-1211 Geneva 4, Switzerland
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Neural actions of several substance P antagonists in the rat spinal cord

Domoto, T., Jury, J., Berezin, I., Fox, J.E.T., Daniel, E.E.
Shimane Med Univ, Dept Anat, Izumo, Shimane 693, Japan
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Bjorkroth, U.
Karolinska Inst, Dept Pharmacol, S-10401 Stockholm 60, Sweden
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Inhibition of smooth muscle contractions induced by capsaicin and electrical transmural stimulation by a substance P antagonist

Kessler, J.A, Adler, J.E., Bell, W.O., Black, I.B.
Cornell Univ, Med Ctr, Coll Med, Div Dev Neurol, New York, NY 10021, USA
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Substance P and somatostatin metabolism in sympathetic and special sensory ganglia in vitro

Uddman R., Malm L., Sundler F.
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BOOKS, REVIEWS, SYMPOSIA

Girod, C.
Gustav Fischer Verlag, Stuttgart, New York, 1983, ISBN: 3-437-10811-5
536 pp, DM 360
Handbuch der Histochemie VIII/5 Supplement. Immunocytochemistry of the Vertebrate Adenohypophysis
Immunocytochemical methods have been used on the pituitary gland since the mid-nineteen fifties and are now indispensible not only for study of pituitary disorders but in any investigation of pituitary cytology and physiology and in the exciting field of research into newly-discovered peptides and their localization and actions within the gland. This book begins with a useful explanation of the most popular immunocytochemical techniques and the unreliability of tinctorial as compared with immuno-cytochemical stains is rightly stressed throughout. However, its main impact lies in the author's comprehensive and analytical review of the vast quantity of published work up to 1982 dealing with application of immunocytochemistry to the adenohypophysis. The bibliography (references are helpfully given in full) occupies a quarter of the book which covers the whole vertebrate kingdom including the foetal as well as the adult gland. One section is devoted to human pituitary tumour pathology. Each chapter ends with a much-needed summary. The author's first language is not English, which occasionally leads to potential misunderstandings (e.g. "weak dilution" for "low dilution" on p.33) but on the whole the text is clear enough. It is complemented by copious footnotes which make for jerky reading, although they are usually interesting. There are plenty of illustrations but the figure legends are sadly lacking in detail. For instance, descriptions of the type of immunoreaction (pre- or post-embedding, fixation, indirect or PAP method) are not given for most of the electron micrographs and neither they nor the light micrographs are provided with magnifications which makes inter-species and inter-cell comparison very difficult. These are relatively minor faults. All in all, this book provides a magnificent compendium of historical and current research on many aspects of the adenohypophysis. Review by Dr J Polak, Royal Postgrad Med Sch, London, England

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Med Coll Penn, Dept Pharmacol, Philadelphia, Pa 19129, USA
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Kuffler, S.W., Sejnowski, T.J.*
*Johns Hopkins Univ, Dept Biophys, Baltimore, md 21218, USA
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Niewoehner, D.E., Levine, A.S., Morley, J.E.*
*Vet Adm Med Ctr, Pulm Sect, Minne -apolis, Mn 55417, USA
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Kommune Hosp, Dept Surg 1, DK-1399 Copenhagen K, Denmark
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Walker J.B.
Pain Inst, Los Angeles, Ca 90025, USA
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Grimmelikhuijzen C.J.P.
Max Planck Inst Med Res, Biophys Abt, D-6900 Heidelberg, W Germany
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Jan, Y.N., Jan, L.Y.
Univ Calif San Francisco, Sch Med, Dept Physiol, San Francisco, Ca 94143, USA
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Centre d'Immunologie, INSERM CNRS de Marseille Luminy, Case 906, 13288 Marseille Cedex 9, France
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Free Univ Brussels, Brugmann Hosp, Neuropathol Neuropeptide Res Lab, Neuropathol Clin, B-1050 Brussels, Belgium
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*St Louis Univ, Med Ctr, Dept Pharmacol, St Louis, Mo 63104, USA
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Dept Med, Westminster Med Sch, Page St Wing, Westminster Hosp, London SW1P 2AP, England
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Berkenbosch, F., Tilders, F.J.H.*, Vermes, I.
*Dept Pharmacol, Med Fac, Free Univ, Van der Boechorststraat 7, 1081 BT Amsterdam, Netherlands
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Kaiya, H., Tanaka, T., Takeuchi, K., Morita, K., Adachi, S., Shirakawa, H., Ueki, H., Namba, M.
Gifu Univ, Sch Med, Dept Neuropsychiat, Gifu 500, Japan
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Pfeiffer, A., Feuerstein, G.*, Zerbe, R.L., Faden, A.I., Kopin, I.J.
*Uniformed Serv Univ Hlth Sci, Neurobiol Res Unit, Bethesda, Md 20814, USA
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Hermansen, K.
Aarhus Univ, Aarhus Kommune Hosp, Internal Med Clin 2, DK 8000 Aarhus C, Denmark
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Leningrad Pediat Med Inst, Dept Pharmacol, Leningrad, USSR
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*Univ Liverpool, Physiol Lab, Liver -pool L69 3BX, Merseyside, England
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NINCDS, Neurootolaryngol Lab, Bldg 36, RM 5D-32, Bethesda, Md 20205, USA
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*Clin Res Inst Montreal, 110 Pine Ave W, Montreal, Quebec, Canada H2W 1R7
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Dept Obstet & Gynaecol, Queen's Univ, Kingston, Ontario, Canada K7L 3N6
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Hosp Sick Children, Res Inst, Toronto, Ontario, Canada M5G 1X8
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*Univ Michigan, Dept Pharmacol, Ann Arbor, Mi 48109, USA
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*State Univ Utrecht, Fac Med, Rudolf Magnus Inst Pharmacol, 3521 GD Utrecht, Netherlands
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Katz, R.J.
Johns Hopkins Univ, Dept Psychol, Baltimore, Md 21218, USA
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Stengaard-Pedersen, K., Fredens, K., Larsson, L.-I.
Aarhus Univ, Inst Med Biochem, DK-8000 Aarhus C, Denmark
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NIMH, Program Intramural Res, Clin Neurosci Branch, Bethesda, Md 20205, USA
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Maier, S.F.
Univ Colorado, Dept Psychol, Boulder, Co 80309, USA
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*Univ Minnesota, Coll Pharm, Dept Med Chem, Minneapolis, Mn 55455, USA
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Univ Oxford, Physiol Lab, Oxford OX1 3PT, England
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Glyclyglutamine, the C-terminal dipeptide of beta-endorphin, occurs in the brain stem and inhibits brain-stem neurones (abstract)

Bornstein, J.C., Costa, M., Furness, J.B., Lees, G.M.
Flinders Univ S Australia, Ctr Neurosci, Bedford Pk, S Australia 5042
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Ramirez-Gonzalez, M.D., Tchakarov, L., Mosqueda, R., Kunos, G.*
*McGill Univ, Dept Pharmacol, Montreal, Quebec, Canada H3G 1Y6
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Beta-endorphin activity on the brainstem is involved in the antihypertensive action of clonidine and alpha-methyldopa in rats

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Fac Sci & Tech Rouen, Endocrinol Lab, CNRS, ERA 891, Rech Endocrinol Molec Grp, F-76130 Mt St Aignan, France
GEN COMP ENDOCRINOL 1983, 51 (2) 246
Immunological characterization of endorphins, adrenocorticotropin, and melanotropins in frog hypothalamus

Joseph, S.A., Pilcher, W.H., Bennett-Clarke, C.
Univ Rochester, Sch Med & Dent, Neuroendocrine Unit, Rochester, NY 14642, USA
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*State Univ Utrecht, Inst Molec Biol, 3508 TB Utrecht Netherlands
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Senba, E., Tohyama, M.
Osaka Univ, Inst Higher Nervous Activ, Dept Neuroanat, Kitaku, Osaka 530, Japan
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Lamotte, C.C., de Lanerolle, N.C.
Yale Univ, Sch Med, Neurosurg Sect, New Haven, Ct 06510, USA
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Ultrastructure of chemically defined neuron systems in the dorsal horn of the monkey. II. Methionine-enkephalin immunoreactivity

Reymann, K.G., Chepkova, A.N., Matthies, H.
Acad Sci GDR, Inst Neurobiol & Brain Res, DDR-3090 Magdeburg, E Germany
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McLean, S., Hoebel, B.G.*
*Princeton Univ, Dept Psychol, Princeton, NJ 08544, USA
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*Fac Med Nord, Med Exptl Lab, CNRS, ERA 982, Blvd Pierre Dramard, F-13326 Marseille 03, France
PEPTIDES 1983, 4 (3) 301
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Mt Sinai Hosp & Med Ctr, Div Endocrinol, New York, NY 10029, USA
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Circadian variation of beta-endorphin-like immunoreactivity in neurointermediate pituitary

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Univ Utrecht, Rudolf Magnus Inst Pharmacol, 3521 GD Utrecht, Netherlands
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Neuropharmacol Lab, Dept Nutr & Food Sci, Massachusetts Inst Technol, Cambridge, Ma 02139, USA
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Univ Tokushima, Fac Pharmaceut Sci, Tokushima 770, Japan
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Synthesis and activity of a newly isolated analgesic pentapeptide, neo-kyotorphin

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Univ Ulm, Zentrum Innere Med Abt Innere Med 1, D-7900 Ulm, W Germany
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Effect of morphine, Leu-enkephalin and beta-casomorphins on basal somatostatin release in dogs

Levina, S., Gordon, R.*
*Univ Manitoba, Fac Med, Dept Pathol, Winnipeg, Manitoba, Canada R3E OW3
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Methionine enkephalin-induced changes in pigmentation of zebrafish (cyprinidae, Brachydanio rerio) and related species and varieties, measured videodensitometrically. I. Zebrafish

Gordon, R., Levina, S.
Address as above
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Methionine enkephalin-induced changes in pigmentation of zebrafish (cyprinidae, Brachydanio rerio) and related species and varieties, measured videodensitometrically. II. Pearl and gold danios

Rodrigues, K., Sumpter, J.P.
Brunel Univ, Sch Biol Sci, Dept Appl Biol, Uxbridge UB8 3PH, Middx, England
GEN COMP ENDOCRINOL 1983, 51 (3) 454
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*Univ Minnesota, Sch Med, Dept Pharmacol, Minneapolis, Mn 55455, USA
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*Erasmus Univ, FAc Med, Dept Pharmacol, 3000 DR Rotterdam, Netherlands
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Correlation between the distribution of 3H-labelled enkephalin in rat brain and the anatomical regions involved in enkephalin-induced seizures

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Karolinska Inst, Dept Histol, S-10401 Stockholm 60, Sweden
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Modulation of nicotinic receptor function by opiate recognition sites highly selective for met5-enkephalin [Arg6Phe7]

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*Univ Georgia, Dept Chem, Athens, Ga 30602, USA
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The synthesis, bioactivity, and enzyme stability of D-Ala2, EPhe4, Leu5-enkephalins

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*Aghia Paraskevi, Nucl Res Ctr Demokritos, Dept Biol, Attiki, Greece
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Duve, H., Thorpe, A.
Univ London Queen Mary Coll, Sch Biol Sci, London E1 4NS, England
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Immunocytochemical identification of alpha-endorphin-like material in neurones of the brain corpus cardiacum of the blowfly, Calliphora vomitoria (Diptera)

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CUNY, Mt Sinai Sch Med, 1 Gustave L Levy Pl, New York, NY 10029, USA
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The multiple faces of pro-opiomelanocortin a prototype precursor molecule

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Karl Marx Univ, Med Branch, Pharmacochem Res Grp, DDR-7010 Leipzig, E Germany
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The effect of opioid drugs on the release of dopamine and 5-hydroxytryptamine from rat striatum following activation of nicotinic-cholinergic receptors

Lo C.W., Wen H.L., Ho W.K.K.*
*Chinese Univ Hong Kong, Dept Biochem, Shatin, Hong Kong
EUR J PHARMACOL 1983, 92 (1-2) 77
Cerebrospinal fluid (Met5)enkephalin level in schizophrenics during treatment with naloxone

Jefferys D., Copolov D., Irby D., Funder J.*
*Prince Henrys Hosp, Med Res Ctr, Melbourne, Victoria 3004, Australia
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Behavioural effect of adrenalectomy: reversal by glucocorticoids or (D-Ala2,Met5)enkephalinamide

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*Kyoto Univ, Sch Med, Dept Med, Div 2, Sakyo Ku, Kyoto 606, Japan
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Plasma methionine-enkephalin and leucine-enkephalin in normal subjects and patients with pheochromocytoma

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*Univ Calif San Francisco, Dept Pediat, San Francisco, Ca 94143, USA
J CLIN ENDOCRINOL METAB 1983, 57 (4) 723
Endocrine, histological and biochemical studies of adrenocorticotropin- producing islet cells carcinoma of the pancreas in child-hood with characterization of proopiomelanocortin

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Dept Biochem, Miyazaki Med College, Kiyotake, Miyazaki 889-16, Japan
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Novel C-terminally amidated opioid peptide in human phaeochromocytoma tumour

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MRC Ctr, Sch Med, MRC, Neurochem Pharmacol Unit, Hills Rd, Cambridge CB2 2QH, England
BRAIN RES 1983, 272 (2) 291
The regional distribution of neurotensin-like immunoreactivity in central and peripheral tissues of the cat

Goldman, R., Bar-Shavit, Z., Romeo, D.*
*Univ Trieste, Ist Chim Biol, I-34127 Trieste, Italy
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Neurotensin modulates human neutrophil locomotion and phagocytic capability

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INRA, Dept Pharmacol, 180 Chemin Tournefeuille, F-31300 Toulouse, France
REGUL PEPTIDES 1983, 6 (3) 197
Effects of intracerebroventricular administration of neurotensin, substance P and calcitonin on gastrointestinal motility in normal and vagotomized rats

Theodorsson-Norheim, E., Rosell, S.
Karolinska Inst, Dept Pharmacol, S-10401 Stockholm 60, Sweden
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Characterization of human plasma neurotensin-like immunoreactivity after fat ingestion

Huidobro-Toro, J.P.
Catholic Univ Chile, Inst Biol Sci, Dept Physiol Sci, Pharmacol Lab, Santiago 1, Chile
NEUROSCI LETT 1983, 38 (3) 309
Non-neuronal excitatory neurotensin receptors on the taenia coli of the guinea pig: lack of influence of tetrodotoxin and dynorphin

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BRAIN RES 1983, 273 (2) 229
Distribution and origins of neurotensin-containing fibers in the nucleus ventromedialis hypothalami of the rat an experimental immunohistochemical study

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Mosaic distibution of neurotensin-like immunoreactivity in the cat striatum

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SUNY Downstate Med Ctr, Dept Surg, Brooklyn, NY 11203, USA
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Sterling Winthrop Res Inst, Rensselaer, NY 12144, USA
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The effects of chronic morphine treatment on neurotensin-induced antinociception

Thor K., Rokaeus A.
Ersta Hosp, Surg Clin, Stockholm, Sweden
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Antroduodenal motor response induced by (Gln4)-neurotensin in man

Thor, K., Rokaeus, A.
Ersta Hosp, Surg Clin, Stokcholm, Sweden
ACTA PHYSIOL SCAND 1983, 118 (4) 136
Studies on the mechanisms by which (Gln4)-neurotensin reduces lower esophageal sphincter (LES) pressure in man

de Quidt M.E., Emson P.C.
MRC, Neurochem Pharmacol Unit, Hills Rd, Cambridge CB2 2QH, England
BRAIN RES 1983, 274 (2) 376
Neurotensin facilitates dopamine release in vitro from rat striatal slices

SUBSTANCE P

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Lab Develop Neurobiol, Depts Neurol & Neurosci, Albert Einstrin Coll Med, 1300 Morris Park Ave, Bronx, NY 10461, USA
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Substance P and somatostatin regulate sympathetic noradrenergic function

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Misch, K.J., Greaves, M.W.
Inst Dermatol, Homerton Grove, London E9 6BX, England
BRIT J DERMATOL 1983, 109 (3) 367
Neuropeptide activity in venous effluent from the hyperaemic upper limb following short-term arterial occlusion (letter)

Berger G.
ANN PATHOL 1983, 3 (3) 264 Peptide immunohistochemistry and neuroendocrine tumors (Abstract)

Iversen L.L.
Merck Sharp & Dohme, Neurosci Res Ctr, Hoddesdon EN11 9BU, England
TREND NEUROSCI 1983, 6 (8) 293
Special issue: Neurotransmitters and their actions: Introduction, Neuropeptides - what next?

Swanson L.W.
Salk Inst Biol Studies, La Jolla, Ca 92037, USA
TKEND NEUROSCI 1983, 6 (8) 294
Neuropeptides - new vistas on synaptic transmission

Griffiths E.C., Slater P., Widdowson P.S.
Univ Manchester, Dept Physiol, Manchester M13 9PT, England
J PHYSIOL LONDON 1983, 342 (Sept) 38P
Effects of opioids, neurotensin and thyrotrophin- releasing hormone on rectal temperature after application to periaqueductal grey region of rat brain (abstract)

Grobecker, H.
Univ Regensburg, Dept Pharmacol, D-8400 Regensburg, W Germany
EUR NEUROL 1983, 22 SUPPL 1 38
Transmitter-peptide coexistence in the central nervous system

Barnstable, D., Jessell, T., Sanes, J., Stevens, C., Robertson, M.
Rockefeller Univ, 1230 New York Ave, New York, NY 10021, USA
NATURE 1983, 306 (5938) 14
How molecular is neurobiology? (News)

Bijlsma, W.A., Schotman, P., Jennekens, F.G.I., Gispen, W.H.*, De Wied, D.
*State Univ Utrecht, Inst Molec Biol, Div Molec Neurobiol, 3584 CH Utrecht, Netherlands
EUR J PHARMACOL 1983, 92 (3-4) 231
The enhanced recovery of sensorimotor function in rats is related to the melanotropic moiety of ACTH/MSH neuropeptides

Schlegel W., Vecsey A., Eberle A.E., Chretien M., Seidah N.G., Zahnd G.R.
Univ Geneva, Dept Med, Fdn Rech Med, CH-1211 Geneva 4, Switzerland
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Peptides of the neurointermediary lobe of rat pituitary stimulate GH secretion in vitro

Louis, J.C., Rougeot, C., Bepoldin, O., Vulliez, B., Mandel, P., Dray, F.*
*URIA, Inst Pasteur, INSERM, U207, 28 Rue Dr Roux, F-75724 Paris, France
J NEUROCHEM 1983, 41 (4) 930
Presence of somatostatin, enkephalins, and substance P-like peptides in cultured neurons from embryonic chick cerebral hemispheres

Haldarmisra, C., Pevet, P.*
*Netherlands Inst Brain Res, 28 Ijdijk 1095 KJ Amsterdam, Netherlands
J NEURAL TRANSM 1983, 57 (1-2) 111
Influence of testosterone on protein peptide secretion as characterized by the formation of granular vesicles in rat and mouse pinealocytes - an invitro study

Ferri GL, Adrian TE, Ghatei MA, Lee YC, Oshaughnessy D, Vezzadini P, Polak JM, Bloom SK//Hammersmith Hosp, Dept Histochem, London W12 OHS, England
ITAL J GASTKOENTEROL 1983, 15 (3) 217
Quantitative distribution of peptide hormones and neuropeptides in the human intestinal wall (Abstract)

Taguchi T, Tanaka K*, Ikeda K, Matsubayashi S, Yanaihara N//*Kyushu Univ, Fac Med, Dept Pathol, Higashi ku, Fukuoka 812, Japan
VIRCHOWS ARCH A PATH ANAT HIS 1983, 401 (2) 223
Peptidergic innervation irregularities in Hirschsprung's disease. Immunohistochemistry - radioimmunoassay

Wied D
ACTA MORPHOL HUNG 1983, 31 (1-3) 159
Pituitary and hypothalamic hormones as precursor molecules of neuropeptides

Hansen BL, Hansen GN, Hagen C, Brodersen P//Inst Med Microbiol, 22 Juliane Maries Vej, DK-2100 Copenhagen O, Denmark
J NEUROIMMUNOL 1983, 5 (2) 171-183
Autoantibodies against pituitary peptides in sera from patients with multiple sclerosis

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Fricker, L.D., Snyder, S.H.*
*Johns Hopkins Univ, Sch Med, Dept Neurosci, Baltimore, Md 21205, USA
J BIOL CHEM 1983, 258 (18) 10950
Purification and characterization of enkephalin convertase, an enkephalin-synthesizing carboxypeptidase

Zioudrou, C., Varoucha, D., Loukas, S., Nicolaou, N., Streaty, R.A., Klee, W.A.
Nucl Res Ctr Demokritos, Dept Biol, Attiki, Greece
J BIOL CHEM 1983, 258 (18) 10934
Photolabile opioid derivatives of D-Ala2-Leu5-enkephalin and their interactions with the opiate receptor

Muthukumaraswamy, N., Day, A.R., Pinon, D., Liao, C.S., Freer, R.J.*
*Virginia Commonwealth Univ, Med Coll Virginia, Dept Pharmacol, Richmond, Va 23298, USA
INT J PEPTIDE PROT RES 1983, 22 (3) 305
Selective ligands for opioid receptors. beta-cyclopropylalanyl containing analogs of enkephalin

Salvadori, S., Marastoni, M., Tomatis, R.*, Sarto, G.
*Univ Ferrara, Ist Chim Farmaceut & Tossicol, I-44100 Ferrara, Italy
FARM ED SCI 1983, 38 (9) 640
Opioid peptides. Structure-activity relationships in [beta-ala4]dermorphin tetrapeptides - V

Sarto, G., Degli Uberti, E.C., Salvadori, S., Tomatis, R.
Univ Ferrara, Ist Farmacol, I-44100 Ferrara, Italy
FARM ED SCI 1983, 38 (9) 647
Opioid peptides. Analgesic activity of potent demorphin tetrapeptides - VI

Jeanrenaud X., Maeder E., Delpozo E., Felber J.P.
Chu Vaudois, Dept Med, Div Endocrinol & Clin Biochem, CH-1011 Lausanne, Switzerland.
ACTA ENDOCRINOL 1983, 104 (1) 85
Effect of an enkephalin-analogue (FK 33-824) on glucose tolerance in man.

Rothman, R., Westfall, T.C.*
*St Louis Univ, Sch Med, Dept Pharmacol, St Louis, Mo 63104, USA
J NEUROBIOL 1983, 14 (5) 341
Further evidence for an opioid receptor complex

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Univ Pittsburgh, Sch Med, Dept Pharmacol, Pittsburgh, Pa 15261, USA
NEUROSCI LETT 1983, 39 (2) 143
The presence of leucine-enkephalin in the sacral preganglionic pathway to the urinary bladder of the cat

Fratta, W., Zivcovic, B., Gessa, G.L.
Univ Cagliari, Inst Pharmacol, I-09100 Cagliari, Italy
NEUROSCI LETT 1983, 39 (2) 161
Phencyclidine decreases methionine-enkephalin content in rat striatum

Chelyshev, Y.A., Gataullin, R.R.
Kazan Med Inst, Dept Histol, Kazan 420012, USSR
NEUROSCI LETT 1983, 39 (2) 165
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Boogaerts M.A., Vermylen J., Deckmyn H., Roelant C., Verwilghen R.L., Jacob H.S., Moldow C.F., Univ Hosp Leuven, B-3000 Leuven, Belgium
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Risch, S.C., Kalin, N.H., Janowsky, D.S., Cohen, R.M., Pickar, D., Murphy, D.L.
Dept Psychiat, Sch Med, Univ California, San Diego, La Jolla, Ca 92093, USA
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Co-release of ACTH and beta-endorphin immunoreactivity in human subjects in response to central cholinergic stimulation

Mosberg, H.I., Hurst, R., Hruby, V.J.*, Gee, K., Yamamura, H.I., Galligan, J.J., Burks, T.F.
*Univ Arizona, Dept Biochem, Tucson, Az 85721, USA
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Bis-penicillamine enkephalins possess highly improved specificity toward delta opioid receptors

Gysin, B., Schwyzer, R.*
*Swiss Fed Inst Technol, Dept Molec Biol & Biophys, CH-8093 Zurich, Switzerland
ARCH BIOCHEM BIOPHYS 1983, 225 (2) 467
Head group and structure specific interactions of enkephalins and dynorphin with liposomes: investigation by hydrophobic photolabeling

Lahm, H-W., Gerber, L.D., Brink, L., Kilpatrick, D.L., Udenfriend, S.*
*Roche Inst Molec Biol, Roche Res Ctr, Nutley, NJ 07110, USA
ARCH BIOCHEM BIOPHYS 1983, 225 (2) 422
Specific polyclonal antibodies to the carboxyl terminus of [Met]enkephalin-Arg6-Gly7-Leu 8

Wienbeck, M., Karaus, M.
Univ Dusseldorf, Med Klin & Poliklin, Klin D, D-4000 Dusseldorf, W Germany
Z GASTROENTEROL 1983, 21 (8) 365
The effects of deoxycholic and ricinoleic acid on the isolated circular muscle of the cat colon are modified by leucine-enkephalin. (German)

Knuth, U.A., Sikand, G.S., Casanueva, F.F., Havlicek, V., Friesen, H.G.
Max Planck Gesell, Klin Forsch Grp Reprod Med, Steinfurter Str 107, D-4400 Munster, W Germany
LIFE SCI 1983, 33 (15) 1443
Changes in beta-endorphin content in discrete areas of the hypothalamus throughout proestrus and diestrus of the rat

Pollerberg, G.E., Costa, T., Shearman, G.T., Herz, A., Reid, L.D.
Max Planck Inst Psychiat, Dept Neuropharmacol, Kraeplinstr 2, D-8000 Munich 40, W Germany
LIFE SCI 1983, 33 (16) 1549
Opioid antinociception and positive reinforcement are mediated by different types of opioid receptors

Govoni, S., Bosio, A., Di Monda, E., Fazzari, G., Spano, P.F., Trabucchi, M.
Univ Brescia, Dept Pharmacol & Therapeut, Brescia, Italy
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Immunoreactive met-enkephalin plasma concentrations in chronic alcoholics and in children born from alcoholic mothers

Griffiths E.C., Longson D., Whittam A.
Univ Manchester, Dept Physiol, Manchester M13 9PT, England
J PHYSIOL LONDON 1983, 342 (Sept) 39P
Biotransformation of dynorphin (1-17) by rat brain peptidases (abstract)

Terzic B., Samardzic R., Micic D., Krstic S.K., Beleslin B.D.
Med Fac Belgrade, Dept Pharmacl, POb 662, YU-11000 Belgrade, Yugoslavia
PERIOD BIOL 1983, 85 (S2) 162
Intestinal opioid receptors and the peristaltic reflux of the guinea-pig isolated ileum (abstract)

Schusdziarra, V., Rewes, B., Lenz, N., Maier, V., Pfeiffer, E.F.
Univ Ulm, Dept Internal Med 1 D-7900 Ulm, W Germany
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Evidence for a role of endogenous opiates in postprandial somatostatin release

Przewlocki, R., Lason, W., Stach, R., Kacz, D.

Polish Acad Sci, Inst Pharmacol, 12 Smetna Str, PL-31343 Cracow, Poland
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Opioid peptides, particularly dynorphin after amygdaloid-kindled seizures

Yoshikawa, K., Hong, J.-S.*
*NIEHS, Behav & Neurol Toxicol Lab, POB 12233, Res Triangle Pk, NC 27709, USA
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The enkephalin system in the rat anterior pituitary: regulation by gonadal steroid hormones and psychotropic drugs

Lavond, D.G., Mauk, M.D., Madden, J., Barchas, J.D., Thompson, R.F.*
*Stanford Univ, Dept Psychol, Stanford, Ca 94305, USA
PHARMACOL BIOCHEM BEHAV 1983, 19 (2) 379
Abolition of conditioned heart-rate responses in rabbits following central administration of [N-MePHe3,-D-Pro4] morphiceptin

Jackson S., Lowry P.J.
St Bartholomews Hosp, Clin Res Ctr, Protein Hormone & Recombinant DNA Unit, London EC1A 7BE, England
NEUROENDOCRINOLOGY 1983, 37 (4) 248
Secretion of pro-opiocortin peptides from isolated perfused rat pars intermedia cells

Ragavan V.V., Wardlaw S.L.*, Kreek M.J., Frantz A.G.
*630 W 168th St, New York, NY 10032, USA
NEUROENDOCRINOLOGY 1983, 37 (4) 266
Effect of chronic naltrexone and methadone administration on brain immunoreactive beta- endorphin in the rat

Delitala G., Grossman A.*, Besser M.
*St Bartholomews Hosp, Dept Endocrinol, London EC1A 7BE, England
NEUROENDOCRINOLOGY 1983, 37 (4) 275
Differential effects of opiate peptides and alkaloids on anterior pituitary hormone secretion

Uberti E.C.D., Trasforini G., Salvadori S., Margutti A., Tomatis R., Kotola C., Bianconi M., Pansini R.
Univ Ferrara, Inst Clin Med, I-44100 Ferrara, Italy
NEUROENDOCRINOLOGY 1983, 37 (4) 280
Stimulatory effect of dermorphin, a new synthetic potent opiate-like peptide, on human growth hormone secretion

Braas K.M., Childers S.R., U'Prichard D.C.*
*Nova Pharmaceut Corp, Wade Ave, POB 21204, Baltimore, Md 21228, USA
J NEUROSCI 1983, 3 (9) 1713
Induction of differentiation increase Met5- enkephalin and leu5-enkephalin content in NG108-15 hybrid cells: An immunocytochemical and biochemical analysis

Farrell, L.D., Harrison, T.S., Demers, L.M.*
*Penn State Univ, Milton S Hershey Med Ctr, Dept Pathol, Hershey, Pa 17033, USA
PROC SOC EXP BIOL MED 1983, 173 (4) 515
Immunoreactive met-enkephalin in the canine adrenal; response to acute hypovolemic stress

Jeanrenaud, X., Maeder, E., Delpozo, E., Felber, J.P.
Chu Vaudois, Dept Med, Div Endocrinol & Clin Biochem, CH-1011 Lausanne, Switzerland
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Effect of an enkephalin-analogue (FK 33-824) on glucose tolerance in man

Lowry, P.J., Silas, L., McLean, C., Linton, E.A., Estivariz, F.E.
Protein Hormone & Recombinant DNA Unit, St Bartholomews Ctr for Clin Res, St Bartholomews Hosp, London EC1A 7BE, England
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Pro-gamma-melanocyte-stimulating hormone cleavage in adrenal gland undergoing compensatory growth

Genazzani A.R., Petraglia F., Facchini V., Facchinetti F.
Univ Modena, Ostet & Ginecol Clin, I-41100 Modena, Italy
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Circulating beta-endorphin levels at various stages of life: possible connections with migraine pathogenesis

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Univ Modena, Ctr Studie Cefalee, Cattedra & Serv Farmacol Clin, I-41100 Modena, Italy
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Circadian variations of proopiocortin-related peptides in children with migraine

Petraglia F., Martignoni E., Sola D., Cicoli C., Facchinetti F., Nappi G., Genazzani A.R.
Univ Modena, Ostet & Ginecol Clin, I-41100 Modena, Italy
CEPHALALGIA 1983, 3 (Suppl 1) 98
Evaluation of central opioid tonus in menstrual migraine

Eybalin M., Cupo A., Pujol R.
CHR St Charles, INSERM, U254, Neurobiol Audit Lab, F-34059 Montpellier, France
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Ultrastructural localization of enkephalin immunoreactivities in the organ of Corti (French)

Horsthemke, B., Hamprecht, B., Bauer, K.
Tech Univ Berlin, Inst Biochem & Molek Biol, D-1000 Berlin 10, W Germany
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Heterogeneous distribution of enkephalin-degrading peptidases between neuronal and glial cells

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Univ Amsterdam, Dept Pharm, Div Pharmaceut Chem, 1018 TV Amsterdam, Netherlands
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Univ Oregon, Dept Chem, Eugene, Or 97403, USA
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Primary structure of the human proenkephalin gene

Nabeshima, T., Yamada, K., Kameyama, T.*
*Meijo Univ, Fac Pharmaceut Sci, Dept Chem Pharmacol, Nagoya, Aichi 468, Japan
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Contribution of different opioid systems to footshock-induced analgesia and motor suppression

Kohno, Y., Tanaka, M., Hoaki, Y., Ida, Y., Nagasaki, N.
Kurume Univ, Sch Med, Dept Pharmacol, Kurume, Fukuoka 830, Japan
EUR J PHARMACOL 1983, 92 (3-4) 265
Differential modification by opioid agents of acutely enhanced noradrenaline release in discrete brain regions

Darlak, K., Grzonka, Z.*, Janicki, P., Czlonkowski, A., Gumulka, S.W.
*Univ Gdansk, Inst Chem, PL-80952 Gdansk, Poland
J MED CHEM 1983, 26 (10) 1445
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Portoghese, P.S., Takemori, A.E.
Univ Minnesota, Coll Pharm, Dept Med Chem, Minneapolis, Mn 55455, USA
J MED CHEM 1983, 26 (10) 1341
Different receptor sites mediate opioid agonism and antagonism

Locatelli, V., Petraglia, F., Penalva, A., Panerai, A.E.
Univ Milan, Sch Med, Dept Pharmacol Chemotherapy & Toxicol, I-20129 Milan, Italy
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Effect of dopaminergic drugs on hypothalamic and pituitary immunoreactive beta-endorphin concentrations in the rat

Houghten, R.A., Bartlett, S.M., Ostresh, J.M.
Scripps Clin & Res Fdn, Res Fdn, Res Inst, Dept Molec Biol, 10666 N Torrey Pines Rd, La Jolla, Ca 92037, USA
LIFE SCI 1983, 33 (18) 1811
alpha-neo-endorphin: receptor binding properties of the tritiated ligand

Wu, K.M., Martin, W.R., Kamerling, S.G., Wettstein, J.G.
Univ Kentucky, Coll Med, Dept Pharmacol, Lexington, Ky 40536, USA
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Possible medullary kappa hyperalgesic mechanism I. A new potential role for endogenous opioid peptides in pain perception

Kamerling, S.G., Martin, W.R., Wu, K.M., Wettstein, J.G.
Univ Kentucky, Coll Med Ctr, Dept Pharmacol, Lexington, Ky 40536, USA
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Izumi, R., Takahashi, M., Kaneto, H.
Nagasaki Univ, Fac Pharmaceut Sci, Dept Pharmacol, Nagasaki 852, Japan
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Involvement of different mechanisms, opioid and non-opioid forms, in the analgesia induced by footshock (FS) and immobilized water immersion (IW) stress

Hoffman, D.W.
NINCDS, Neurootolaryngol Lab, Bldg 36, Room 5D08, Bethesda, Md 20205, USA
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Chromatographic identification of enkephalins in the guinea pig retina

Szczudlik A, Lypka A//Ctr Postgrad Med Educ MMA, Dept Neurol, Pain Res Lab, Warsaw, Poland
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Plasma immunoreactive beta-endorphin and enkephalin concentration in healthy subjects before and after electroacupuncture

Linari MB, Castellacci R, Linari G*//*Univ Rome, Ist Farmacol Med 1, I-00100 Rome, Italy
ITAL J GASTROENTEROL 1983, 15 (3) 177
The effect of dermorphin on exocrine pancreatic secretion of the rat

Stanghellini V, Malagelada JR//Mayo Clin & Mayo Fdn, Gastrointestinal Unit, Rochester, Mn 55901, USA
ITAL J GASTROENTEROL 1983, 15 (3) 212
Digestive motility and beta-endorphin plasma levels in man, under the effect of stress (Abstract)

Thomas TA, Fletcher JE//Bristol Matern Hosp, Sir Humphrey Davy Dept Anaesthesia Matern, Bristol BS2 8EG, England
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Altstein M, Bachar E, Vogel Z*, Blumberg S//*Weizmann Inst Sci, Dept Neurobiol, POB 26, IL-76100 Rehovot, Israel
EUR J PHARMACOL 1983, 91 (4) 353
Protection of enkephalins from enzymatic degradation utilizing selective metal-chelating inhibitors

Moerschbaecher JM, Thompson DM//Louisiana State Univ, Med Ctr, Dept Pharmacol & Exptl Therapeut, New Orleans, La 70112, USA
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Differential effects of prototype opioid agonists on the acquisition of conditional discriminations in monkeys

Yonehara N, Kudo T, Iwatsubo K, Maeda S, Saito K, Inoki R*//*Osaka Univ, Sch Dent, Dept Pharmacol, Osaka 530, Japan
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A possible involvement of the central endorphin system in the autoanalgesia induced by chronic administration of Freund's adjuvant solution in rats

Furuhashi N, Takahashi T, Fukaya T, Kono H, Shinkawa O, Suzuki M//Tohoku Univ, Sch Med, Dept Obstet & Gynecol, Sendai, Miyagi 980, Japan
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Human plasma ACTH, beta-lipotropin and beta-endorphin during delivery

Rasmussen DD, Liu JH, Wolf PL, Yen SSC*//*Univ Calif San Diego, Sch Med T-002, Dept Reprod Med, La Jolla, Ca 92093, USA
J CLIN ENDOCRINOL METAB 1983, 57 (5) 881
Endogenous opioid regulation of gonadotropin-releasing hormone release from the human fetal hypothalamus in vitro

Miralles FS, Olaso MJ, Fuentes T, Lopez F, Laorden ML, Puig MM//CSV Arriaca Hosp, Dept Anesthesiol, Murcia, Spain
ANESTHESIOLOGY 1983, 59 (4) 366
Presurgical stress and plasma endorphin levels (Letter)

Ermisch A, Ruhle HJ, Neubert K, Hartrodt B, Landgraf R//Karl Marx Univ, Dept Cell Biol & Regulat, Biosci Sect, DDR-7010 Leipzig, E Germany
J NEUROCHEM 1983, 41 (5) 1229
On the blood-brain barrier to peptides: [3H]beta-casomorphin-5 uptake by eighteen brain regions in vivo

McLawhon RW, Cermak D, Ellory JC, Dawson G*//*Univ Chicago Hosp & Clin, Dept Pediat, Chicago, Il 60637, USA
J NEUROCHEM 1983, 41 (5) 1286
Glycosylation-dependent regulation of opiate (enkephalin) receptors in neurotumor cells

Gaillard-Plaza G, Lopez-Timoneda F, Montastruc JL*//*Fac Med Toulouse, Pharmacol Med Clin Lab, 37 Allee Jules Guesde, F-31000 Toulouse, France
REV NEUROL 1983, 139 (8-9) 529
Endorphins and experimental analgesic effect of amitriptyline (French)

Govoni S, Missale C, Castelletti L, Spano PF, Trabucchi M*//*Univ Brescia, Sch Med, Dept Pharmacol & Therapeut, I-25100 Brescia, Italy
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Decreased content of met-enkephalin-like peptides in superior cervical and coeliac ganglia of aged rats

NEUROTENSIN

Theodorsson-Norheim, E., Oberg, K., Rosell, S., Bostrom, H.
Karolinksa Inst, Dept Pharmacol, S-10401 Stockholm 60, Sweden
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Neurotensinlike immunoreactivity in plasma and tumor tissue from patients with endocrine tumors of the pancreas and gut

Bates T.E., Blackhall N.W., Bloom S.R., Farrar G.B.J., Hall J.C., Kinsman R., Lee Y., MacFarlane A., Morris A.P., Moss G., O'Neill B., Read N.W., Welch I.
Univ Sheffield, Dept Physiol, Sheffield S10 2TN, England
J PHYSIOL LONDON 1983, 342 (Sept) 27P
The effect of infusion of nutrient solutions into the ileum on gastrointestinal transit and plasma levels of neurotensin and enteroglucagon (abstract)

Rokaeus A., Al-Saffar A.
Karolinska Inst, Dept Pharmacol, S-10401 Stockholm 60, Sweden
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The importance of bile and pancreatic juice for fat-induced release of neurotensin-like immunoreactivity (NTLI) from the small intestine of the rat

Lee TF, Hepler JR, Myers RD*//*Univ N Carolina, Sch Med, Dept Psychiat, Chapel Hill, NC 27514, USA
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Evaluation of neurotensin's thermolytic action by ICV infusion with receptor antagonists and a Ca++ chelator

SUBSTANCE P

Garciasevilla, J.A., Magnusson, T.,* Carlsson, A., Folkers, K.
*Univ Goteborg, Dept Pharmacol, S-40033 Goteborg, Sweden
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Substance-P - structural requirements for the activation of brain catecholamine synthesis and locomotor activity in rats

Yu, J.H., Burns, S.M., Schneyer, C.A.
Univ Alabama, Dept Physiol & Biophys, Birmingham, Al 35294, USA
PROC SOC EXP BIOL MED 1983, 173 (4) 467
Salivary secretion induced by substance P

Jonsson G. Hallman H.
Karolinska Inst, Dept Histol S-10401 Stockholm 60 Sweden.
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Univ Coll London, Inst Ophthalmol, Dept Visual Sci, London WC1, England
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Joseph, A.B., Bekkemeyer, H., Oehme, P., Foreman, J.C.
St Elizabeths Hosp, Dept Psychiat, Boston, Ma 02135, USA
J ROY SOC MED 1983, 76 (9) 801
Accidental injection of substance P (Letter)

Bynke, G., Hakanson, R., Horig, J.
Univ Lund, Dept Exptl Opthalmol, S-22362 Lund, Sweden
EXPERIENTIA 1983, 39 (9) 996
Ocular responses evoked by capsaicin and prostaglandin E2 are inhibited by a substance P antagonist

Dalsgaard, C.J., Jonsson, C.E., Hokfelt, T., Cuello, A.C.
Karolinska Inst, Dept Anat, S-10401 Stokcholm 60, Sweden
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Localization of substance P-immunoreactive nerve fibers in the human digital skin

Mandahl, A.K., Bill, A.
ACTA OPHTHALMOL 1983, 61 (4) 687
Substance P-release and effects in the rabbit eye. (Abstract)

Lavielle, S., Chassaing G., Marquet A
Univ Paris 06, Chim Organ Biol Lab, ERA 823, F-75230 Paris 05, France
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Avidin binding of biotinylated analogues of substance P

Payan, D.G., Brewster, D.R., Goetzl, E.J.
Univ California, Howard Hughes Med Inst Labs, San Francisco, Ca 94143, USA
J IMMUNOL 1983, 131 (4) 1613
Specific stimulation of human T lymphocytes by substance P

Ekstrom J., Mansson B., Tobin G.
Univ Lund, Dept Physiol & Biophys, S-22101 Lund, Sweden
ARCH ORAL BIOL 1983, 28 (9) 859
Relative secretory contributions of the 3 major salivary glands of the rat in response to substance-P and super-sensitivity

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